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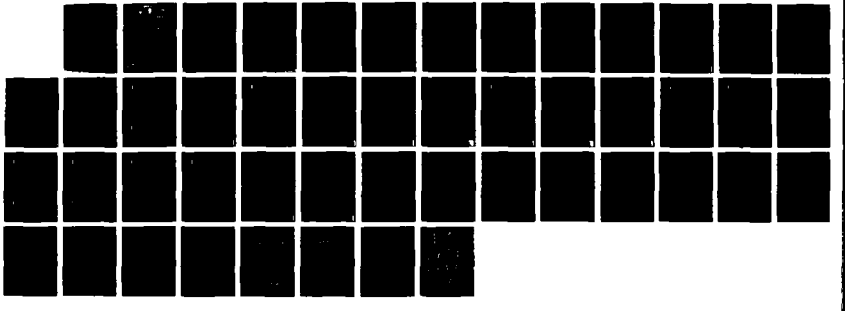

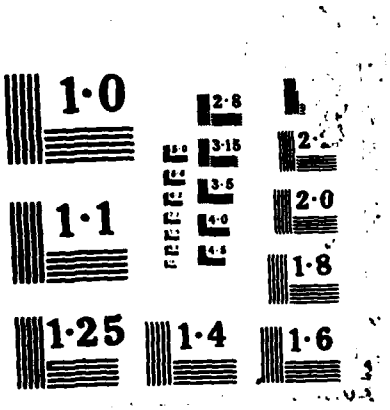
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Significantly greater weight loss was observed in PFDA-treated rats than in pair controlled rats. These data indicate that the wasting syndrome cannot be explained entirely by lack of food intake. Additionally, a precipitous fall in serum thyroxine was observed as early as 12 hours following PFDA. Early experiments indicated that a hypothyroid-like state resulted from PFDA treatment. PFDA induced decreases in serum thyroid hormones, anorexia, bradycardia, and hypothermia, as well as alterations in myocardial catecholamine metabolism. Liver enzymes α-glycerolphosphate dehydrogenase and malic enzyme were measures to evaluate tissue thyroid state, the activity of both enzymes was significantly elevated as early as 24 hours and remained so throughout the experiment.

A possible conclusion based on interpretation of these data is that PFDA alters biochemical processes at the cellular level which produces confused messages concerning metabolic status, thus leading to anorexia and metabolic inefficiency which results in severe body wasting and hypothermia.

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Anoretic response to PFDA

We have made several observations which suggest a possible mechanism for the anoretic effect of PFDA. In rats fasted overnight PFDA-treatment produced a dose-related increase in blood glucose which was significantly greater than control 24 hours after dosing (Table 1). The activation of hepatic vagal afferents by increased blood glucose[7] and the detection of high blood glucose levels by glucose-sensitive fibers in the LH[9] and/or ventral medial hypothalamus[14] could initiate mechanisms to reduce blood glucose. One of these mechanisms is decreased food intake. The observation that T_4-treatment increased fasted blood glucose levels (Table 1) but did not reduce food consumption would appear to be evidence against this hypothesis. In fact T_4 pretreatment inhibited the anoretic response to PFDA (Figure 1). This is not inconsistent with the hypothesis if one considers the role of thyroid releasing hormone (TRH) in the hypothalamic modulation of feeding behavior. PFDA has been shown to rapidly and dramatically reduce serum thyroxine levels which results in a reflex increase in thyroid stimulating hormone (TSH)[15] and perhaps also TRH secretion[1]. Treating PFDA-dosed rats with T_4 may restore the inhibition of TRH secretion. This suggests that if they are separate mechanisms the central TRH phenomenon can override the peripheral hyperglycemic signal transmitted via hepatic vagal afferents to the hypothalamus or if they are parts of the same mechanism the site of the TRH effect is beyond that of hepatic vagal input.

TABLE 1

24-HOUR DOSE RESPONSE RELATIONSHIP WITH PFDA TREATMENT AND SERUM GLUCOSE

PFDA Dosage (mg/kg)	N	Glucose[a,b] (gm/dl)
Control	2	75.50 ± 2.50
6.25	3	75.33 ± 3.84
10[c]	2	107.00 ± 1.00*
12.5	5	79.00 ± 2.35
25	5	87.60 ± 6.24
50	5	88.00 ± 3.86*
100	5	106.10 ± 2.76*
75 [illegible]	5	135.00 ± 5.51*

[a] Fasting glucose levels collected 24 hours after a single injection of PFDA

[b] Values are X ± SEM

[c] Rats were treated with 200 mg/kg thyroxine daily for 10 days

* Significantly different from controls, $p<0.05$, Student's t test

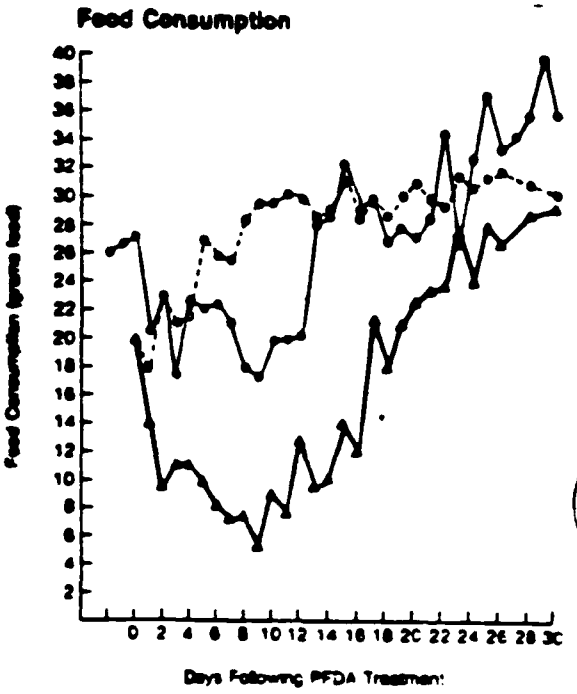

Figure 1 Food consumption following treatment with T_4, ○ (n=8); PFDA, ▲ (n=8); or T_4 + PFDA, ● (n=8).

With recognition of the important role of the ventral medial nucleus of the hypothalamus in feeding behavior we conducted a pilot study designed to examine the effect of lesioning of this area on PFDA-induced anorexia. Systemic administration of the glucose analog gold thioglucose produces brain lesions which are localized to the ventral medial nucleus. Rats were fasted for 24 hours prior to receiving a single dose of 12.0 mg/kg gold thioglucose (GTG). The animals were allowed to recover from the GTG

for 7 days at which time half of the animals received a single 75 mg/kg dose of PFDA and the remaining animals received propylene glycol. Food consumption and body weight changes were monitored for a period of 30 days. The average daily food consumption over the first eight days following PFDA treatment was greater in the GTG pretreated rats than in the rats treated with PFDA alone (Figure 2). During this period the GTG plus PFDA treated rats lost less body weight than rats treated with PFDA alone although the difference was not significant. The animals were observed for an additional 30 days at which time they were sacrificed and serum levels of T_3 and T_4 were examined. The effect of PFDA alone on T_4 was still present 60 days after exposure while the effect on T_3 showed recovery. GTG treatment alone produced increased T_4 levels with no apparent change in T_3 while GTG treatment prior to PFDA prevented the long-lasting fall in T_4 seen with PFDA alone. These preliminary data suggest that GTG lesions of the ventral medial hypothalamus can partially prevent the anoretic response to PFDA but are ineffective at preventing the body weight loss. In addition GTG pretreatment prevents or reverses the long-lasting fall in thyroxine produced by PFDA.

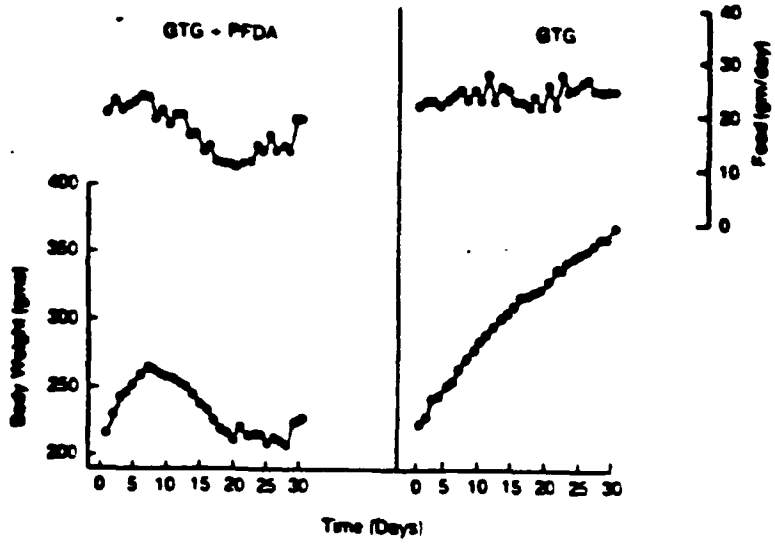

Figure 2 Effect of gold thioglucose (GTG) on body weight (●) and food consumption (○) responses to PFDA. GTG, n=2; GTG + PFDA, n=3.

Fatty acid oxidation by the liver can also reduce food intake by acting via hepatic vagal afferents to the hypothalamus.[3] This is most apparent during periods of high lipid metabolism.[17] PFDA-treatment alters mechanisms that affect the composition and/or metabolism of fatty acids in the liver and heart. We have observed changes in the relative percentages of several fatty acids in heart homogenates[12] and similar effects have been reported in liver homogenates[8] from PFDA-treated rats. In addition, several investigators have reported proliferation of liver peroxisomes important extramitochondrial sites of fatty acid oxidation and a 20-40 fold increase in fatty acyl CoA enzyme activity in rats treated with PFDA. These observations relate to the hypothesis of Scharrer and Langhans[17] concerning alterations in fatty acid oxidation leading to changes in the production of reducing equivalents and their effect on food intake.

We observed significantly greater weight loss in PFDA-treated rats than in pair-fed control rats.[4] Our results are similar to those reported by Olson and Andersen.[8] These data indicate that the wasting syndrome cannot be entirely explained by the lack of food intake. Additionally we observed a precipitous fall in serum thyroxine as early as 12 hours following PFDA.[4] A more detailed examination of the effects of PFDA on the thyroid system has led to a number of observations. The early experiments suggested that a hypothyroid-like state resulted from PFDA-treatment. PFDA-induced decreases in serum thyroid hormones, anorexia, bradycardia, and hypothermia[4] as well as alterations of myocardial beta-receptor binding characteristics and decreased adenylate cyclase activation by catecholamines[11,12] led to the tentative conclusion that many of the toxic effects of PFDA could be explained by a chemically induced hypothyroidism. Experiments employing T_4-supplementation suggests that the toxicity is more complex. Rats were treated with thyroxine (200 mg/kg) for 10 days, dosed with PFDA on the eleventh day and sacrificed 14 days after PFDA treatment. Thyroxine supplementation was continued through the day prior to sacrifice. The liver enzymes α-glycerolphosphate dehydrogenase (GPD) and malic enzyme (ME) were measured one, seven, and 14 days after PFDA in order to evaluate the thyroid state of the animals at the tissue level. Glycerol-phosphate dehydrogenase is involved in shuttling reducing equivalents from cytosolic NADH into the mitochondrial electron transport chain[6] and malic enzyme is involved in lipogenesis and saturation and elongation of fatty acids.[6] The activity of both enzymes is increased in hyperthyroidism,[10] and decreased in hypothyroidism.[16] In our experiments the activity of both enzymes was significantly elevated by PFDA as early as 24 hours after treatment and remained elevated at 7, and 14 days after treatment (Table 2). Rats treated for 10 days with T_4 prior to receiving PFDA had enzyme activities which were greater than either treatment alone at most time points. The effects on glycerolphosphate dehydrogenase appear to be additive while the effects on malic enzyme are supra-additive at 7 and 14 days. These data are difficult to interpret in terms of the thyroid state of the animals since the results are opposite to what would be expected if PFDA produces a true hypothyroidism. The observations are significant in view of the PFDA-induced starvation syndrome. Starvation normally leads to a mobilization of fatty acids as metabolic fuels for energy production.[5] Consistent with PFDA-induced starvation Pilcher et al.[13] reported increased capacity for hepatic fatty acid oxidation through activation of a peroxisomal pathway at 2 and 8 days after PFDA. However, Kelling et al.[2] reported increases in the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme 7 days following PFDA treatment. The activities of these enzymes provide most of the NADPH required for fatty acid biosynthesis.[5] These data indicate that the capacity for fatty acid metabolism is enhanced either directly or secondary to starvation and paradoxically the capacity for fatty acid biosynthesis is also increased by PFDA treatment. The latter is an inappropriate response and supports the hypothesis that the generation and/or interpretation of internal signals concerning metabolic status are confused in PFDA-treated animals.

Table 2

Activities of alpha-glycerophosphate dehydrogenase (GPD) and malic enzyme (ME) in rat liver

GPD Activity
(nmol substrate/min/mg protein)

	24 HRS	7 DAYS	14 DAYS
Control	57.7 ± 9.3	53.1 ± 3.4	57.8 ± 13.9
Pair-fed Control	---	42.9 ± 2.9	95.4 ± 16.7
T_4	105.6 ± 2.0	113.3 ± 2.9	122.1 ± 15.5
PFDA	99.7 ± 33.2	109.6 ± 6.6	285.1 ± 11.1
T_4 + PFDA	238.2 ± 4.2	220.6 ± 24.2	344.4 ± 53.9

ME Activity
(nmol substrate/min/mg protein)

Control	2.42 ± 1.2	2.57 ± 1.6	1.56 ± 1.2
Pair-fed control	---	3.8 ± 0.6	undetectable
T_4	36.1 ± 5.4	25.3 ± 4.5	24.3 ± 7.1
PFDA	6.9 ± 1.6	25.7 ± 7.3	12.7 ± 3.0
T_4 + PFDA	36.0 ± 3.1	113.0 ± 21.3	158.0 ± 22.6

The most significant results of similar experiments carried out for 30 days were the observations that T_4 supplementation had differential effects on anorexia, body wasting and hypothermia. Thyroxine supplementation completely prevented the PFDA-induced anorexia (Figure 1) but had no effect on the wasting syndrome (Figure 3) or the hypothermia (Figure 4). These results suggested to us that critical metabolic processes associated with energy production (and maintenance of body mass) as well as thermogenesis were severely altered by this halogenated chemical. Because these processes are dependent on normal mitochondrial function many of the earlier observations which we ascribed to a PFDA-induced hypothyroidism can be interpreted in terms of time dependent alterations of cellular metabolic processes most likely in mitochondria.

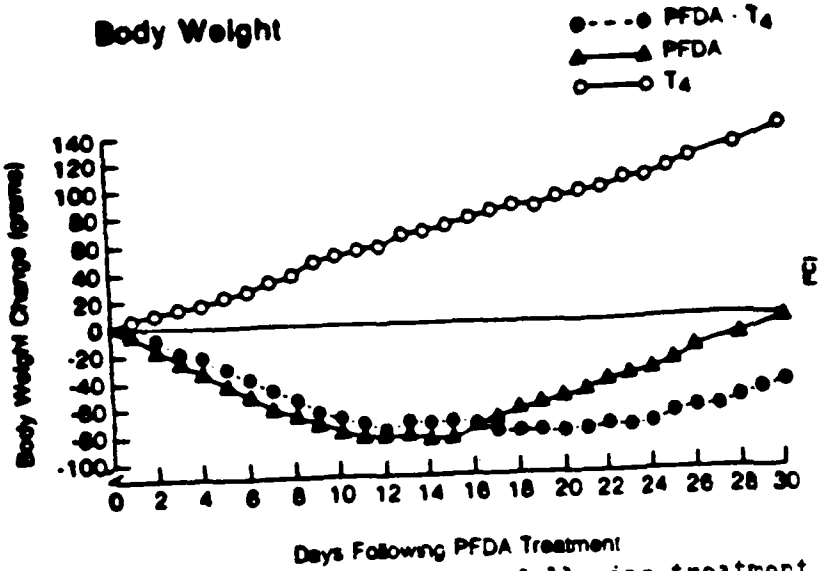

Figure 3 Body weight changes following treatment with T_4, ○ (n=8); PFDA, ▲ (n=8); or T_4 + PFDA, ● (n=8).

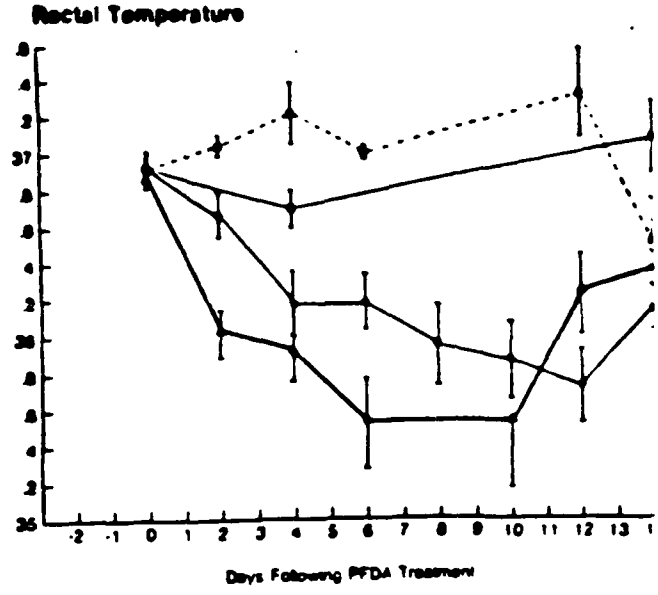

Figure 4 Rectal body temperatures following treatment with T_4, ○ (n=8); PFDA ▲ (n=8); or T_4 + PFDA, ● (n=8).

In the early stages of starvation body proteins are broken down for gluconeogenesis in order to provide glucose for brain metabolism. In long-term starvation in addition to metabolizing glucose the brain adapts for metabolism of ketone bodies especially 8-hydroxybutyrate in order to spare body proteins.[5] The PFDA-induced disruption of mitochondrial function would lead to a number of responses that could account for some of the observations that we have made. The mobilization of fatty acids as a result of starvation causes proliferation of peroxisomes as an extramitochondrial pathway for metabolism. A major portion of the acetyl Co A formed in the cytosol from this process is converted to acetoacetate because of slowing of the Krebs Cycle at the succinate dehydrogenase step. In addition, hydroxybutyrate formation from acetoacetate is inhibited by PFDA-induced alterations of mitochondrial function so that brain activity continues to rely on glucose supplied by gluconeogenesis from amino acids as a result of further breakdown of body proteins stimulated in part by glucocorticoids.

The high level of NADH formed in the cytosol by peroxisomal fatty acid oxidation may account for the paradoxical increase in glycerolphosphate dehydrogenase activity that we observed (Table 2). This may be a compensitory mechanism attempting to shuttle the additional reducing equivalents into mitochondria.[5]

A possible conclusion based on the interpretation of these data is that PFDA alters biochemical processes at the cellular level which produces confused messages concerning metabolic status leading to anorexia and metabolic inefficiency resulting in severe body wasting and hypothermia.

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THYROID, BRADYCARDIC AND HYPOTHERMIC EFFECTS OF PERFLUORO-n-DECANOIC ACID IN RATS

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A single ip injection of perfluoro-n-decanoic acid (PFDA) to male Wistar rats resulted in an initially rapid, then gradual decrease in food consumption and a parallel loss of body weight. Body temperatures and resting heart rates were significantly depressed by PFDA treatment. As early as 12 h following a single dose of PFDA, serum thyroxine (T_4) levels were significantly reduced and remained depressed throughout the 8 day study. Serum triiodothyronine (T_3) was reduced by 35% 12 h following PFDA treatment and remained at that level throughout the study. These preliminary data suggest that an action on the thyroid axis may be an early primary response to PFDA and that some of the observed subsequent effects may in part be secondary to the change in thyroid hormone levels.

INTRODUCTION

Perfluoro-*n*-decanoic acid (PFDA) is a member of a family of perfluorocarboxylic acids that are used in a variety of industrial applications. These include film-forming foams for fire extinguishants, wetting agents, corrosion inhibitors, and for electroplating (Olson, 1983). They have also been used to impart water and oil resistance to leather, certain fabrics, and food wrapping paper (Rozner, 1980).

There have been reports of human exposure to perfluorinated carboxylic acids primarily in workers in fluorochemical plants (Ubel et al., 1980). The study demonstrated that these compounds persisted in the body long after exposure had been discontinued. While no apparent toxicities were reported with these acute exposures, the persistent nature of this class of chemicals may produce long-term effects.

Toxicity has been reported in laboratory animals, especially the rat. The gross toxicity produced by PFDA includes hypophagia, dramatic weight loss, and delayed lethality. The LD50 in male Fisher-344 rats was 64 mg/kg (Olsen 1983). The LD50 in male Wistar rats is around 75 mg/kg (M. E. Andersen, personal communication). The acute gross toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is also characterized by hypophagia, dramatic weight loss, and a delayed lethality (Seefeld and

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Peterson, 1982). Recently, Potter et al. (1983) showed that TCDD treatment produced a significant reduction in body temperature and a decrease in serum thyroxine (T_4) levels. Because of the similarity in the gross toxicity of PFDA to that of TCDD (Andersen, 1981), it was of interest to investigate the effect of PFDA on body temperature and serum thyroid hormone levels. In addition, the effect of PFDA on resting heart rate was also measured.

METHODS

Groups of male Wistar rats (200-250 g) were obtained from Harlan Industries and maintained in a constant-temperature environment (23.3°C, range 22.1-24.4°C) for 6 d prior to use. PFDA-treated animals received a single 75-mg/kg ip injection of PFDA in propylene glycol. One group of 30 rats was treated with PFDA at 5:00 p.m. on d 0, and daily food consumption and body weights were recorded over a period of 8 d. At 1 d after the PFDA-treated rats were dosed, a group of 30 weight-matched control rats was injected with propylene glycol at 5:00 p.m., pair-fed to the PFDA-treated group, and body weights were recorded. A group of 8 rats fed ad libitum were injected with propylene glycol at 5:00 p.m. on d 0 and their food consumption and body weights recorded for 8 d. All animals were weighed between 4:00 and 5:00 p.m. daily and then offered food. At different intervals beginning 12 h after dosing (5:00 a.m.), five rats (PFDA-treated or pair-fed) were sacrificed by decapitation and blood was collected in centrifuge tubes on ice, allowed to coagulate, and centrifuged at 3000×g for 10 min. The serum was stored at −20°C for triiodothyronine (T_3) and T_4 determinations. Ad libitum control rats were also sacrificed on d 0, 1, and 2 for determination of serum T_3 and T_4.

Rectal body temperatures were recorded at 2:00 p.m. daily for 8 d in a group of 17 PFDA-treated, 17 pair-fed control rats, and 8 ad libitum controls. A rectal thermometer was inserted to a depth of 3 cm, and the temperature was recorded on a Yellow Springs Instrument Company telethermometer.

Separate groups of eight PFDA-treated and eight pair-fed control rats were used to determine heart rates. Between 4:00 and 5:00 p.m. on alternate days beginning 2 d after dosing, the rats were lightly anesthetized with ether and needle electrodes were inserted under the skin of both front limbs and the right hind limb. The electrodes were connected to an ECG/Biotach Amplifier on a Gould 2400 Physiological recorder, and lead I of the electrocardiogram and instantaneous heart rate were recorded. Stable periods of heart rate recorded as the animals were beginning to emerge from the anesthesia were reported.

Total T_4 and total T_3 were determined by radioimmunoassay using assay tubes coated with antibody to T_4 or T_3 (Diagnostic Products Corpo-

ration, Los Angeles, Calif.). Concentrations of PFDA up to 100 μg/ml did not interfere with the radioimmunoassays for either T_4 or T_3.

All animals were handled in identical fashion and were fasted for 24 h prior to sacrifice. When sacrifice of PFDA-treated and control rats occurred on the same day, the sacrifice routine was randomized.

An analysis of variance (ANOVA) was carried out on the data to determine statistical differences among the groups. A Duncan's multiple range test was used to determine significant differences at the 0.05 level between pair-fed controls and PFDA-treated rats.

RESULTS

Food consumption decreased rapidly in the first 24 h and tapered gradually to 0 g/d by d 8 (Fig. 1). Body weights of the PFDA-treated rats fell from a pretreatment average of 250 g to 160 g at 8 days following PFDA treatment (Fig. 2). The pair-fed group of rats showed a similar weight loss with final weights around 190 g (Fig. 2).

One day following treatment the body temperatures of PFDA-dosed rats were significantly higher than pair-fed controls. Beginning on d 3 and continuing through the end of the study, the body temperatures of PFDA-treated rats were significantly lower than the body temperatures of pair-fed control rats (Fig. 3).

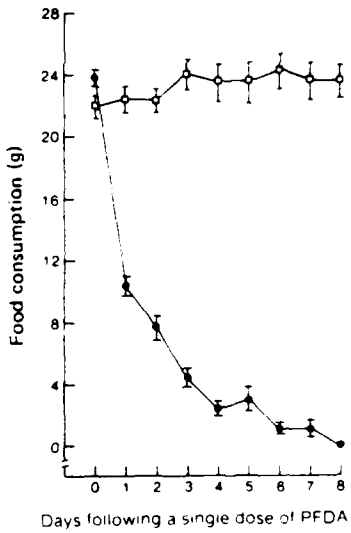

FIGURE 1. The effect of PFDA (75 mg/kg) on the amount of standard laboratory food (g) consumed by rats during a 24-h period. □, control, $n = 8$; ●, PFDA, each point representing the mean ± standard error of the mean (SEM) of a variable number of animals as follows: d 0 = 30, 1 = 25, 2 and 3 = 20, 4 and 5 = 15, 6 and 7 = 10, and 8 = 5.

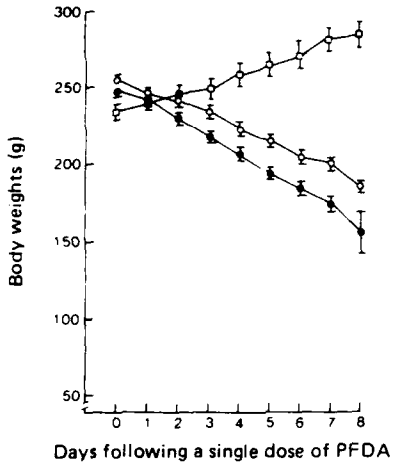

FIGURE 2. The effect of PFDA (●) and food restriction (pair-feeding, ○) on body weights of rats. Each point represents the mean ± SEM of a variable number of animals. Refer to legend of Fig. 1 for details. Ad libitum controls □, $n = 8$.

Food restriction in the pair-fed controls did not alter resting heart rates. PFDA-treated rats showed a progressively lower resting heart rate with time after dosing. By d 6 the recorded heart rates were significantly lower in the PFDA-treated rats than in pair-fed controls (Fig. 4).

As early as 12 h following dosing with PFDA, serum thyroxine (T_4) levels were significantly reduced compared to pair-fed controls (Fig. 5). Serum T_4 levels continued to fall reaching a minimum value by d 2 and continuing at that level through d 8. Serum T_4 levels were significantly lower than pair-fed controls at all tested times.

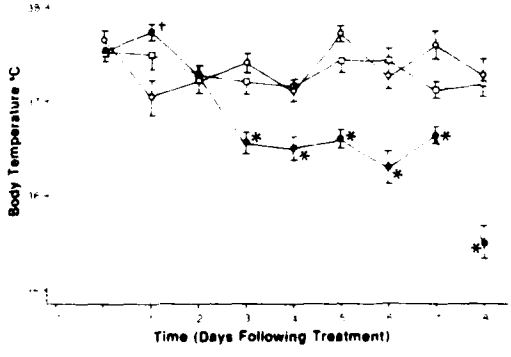

FIGURE 3. The effect of PFDA (●, $n = 17$) and pair-feeding (○, $n = 17$) on rectal body temperatures of rats. *, significantly lower than pair-fed controls, $p < 0.05$. †, significantly higher than pair-fed controls, $p < 0.05$; □, ad libitum controls, $n = 8$.

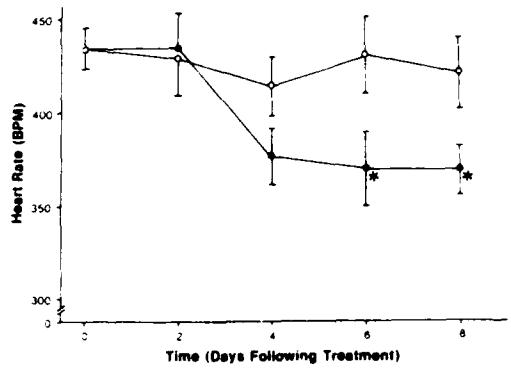

FIGURE 4. The effect of PFDA (●, $n = 8$) and pair-feeding (○, $n = 8$) on resting heart rates in rats. Controls, (□, $n = 16$). *Significantly less than pair-fed control, $p < .05$.

Serum triiodothyronine (T_3) showed a similar but less dramatic response to PFDA treatment (Fig. 6). By 12 h, circulating levels of T_3 were reduced by about 35% in PFDA-treated rats compared to pair-fed controls (72 versus 110 ng/dl). Serum T_3 values remained reasonably constant until d 8, when they fell to 50 ng/dl. Serum T_3 values were significantly lower than pair-fed controls only at 12 h, 1 d, and 2 d.

DISCUSSION

A single ip injection of PFDA (75 mg/kg) produced hypophagia and dramatic weight loss similar in magnitude but with a shorter time course than that previously reported with PFDA at 50 mg/kg (Olson and Andersen, 1983). Food restriction by pair-feeding a group of control rats produced a similar weight loss.

Significant decreases in body temperatures and heart rates occurred in the PFDA-treated rats when compared with pair-fed controls. The data suggest that PFDA may be affecting basic endogenous mechanisms

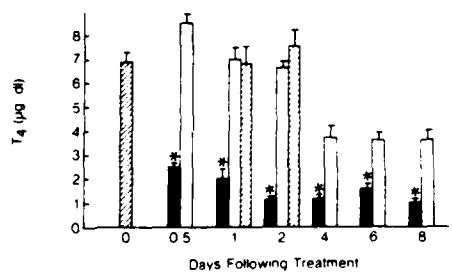

FIGURE 5. Effect of PFDA (solid columns) and pair-feeding (open columns) on rat serum thyroxine (T_4). Each bar is the mean ± SEM of five serum samples. Ad libitum controls (hatched column, d 0, $n = 15$; d 1, $n = 5$; d 2, $n = 5$). *Significantly less than pair-fed controls, $p < 0.05$.

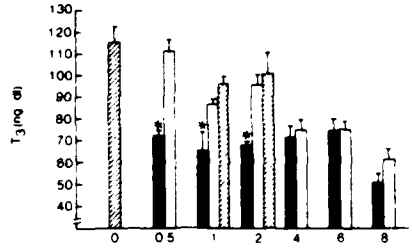

FIGURE 6. Effect of PFDA (solid columns) and pair-feeding (open columns) on rat serum triiodothyronine (T_3). Refer to legend of Fig. 5 for details.

that regulate these physiological functions. Hypothyroid individuals have cold extremities and are cold-sensitive (Ibbertson, 1979), probably due to metabolic alterations that decrease the rate of heat production (Mazzaferri, 1980). The thyroid hormones are reported to influence thermogenesis through an action on ATPase-mediated active sodium transport (Edelman and Ismail-Beigi, 1974; Himms-Hagen, 1976). In addition, thyroid status can affect cardiovascular function. Impaired myocardial contractility (Buccino et al., 1967) and bradycardia (Mazzaferri, 1980) are common manifestations of hypothyroidism. Therefore, we felt that many of the symptoms of PFDA treatment might involve alterations of thyroid hormone levels. Figures 5 and 6 show that as early as 12 h after a single dose of PFDA serum levels of thyroxine and triiodothyronine are significantly lower than controls. This occurs at a time when the animals do not demonstrate any overt signs of PFDA toxicity. Following 4 d of pair-feeding, serum T_3 values were at the same level as that produced by PFDA treatment; however, serum T_4 levels in PFDA-treated rats were significantly lower than in those pair-fed throughout the study. These data indicate that the depression of thyroid hormone levels produced by PFDA is not solely a result of starvation. It is nearly impossible to control all of the numerous influences that can alter circulating levels of thyroid hormones. However, since both groups of animals were handled identically, the fact that serum levels of both thyroid hormones are dramatically decreased as early as 12 h following a single ip injection of PFDA suggests that this may be an important observation in the response to PFDA treatment. The subsequent changes in body temperature and heart rate may be at least partly secondary to the alterations of circulating thyroid hormones. It is not clear from this study whether PFDA is acting directly on the thyroid gland or on the pituitary gland to depress TSH or on the hypothalamus to depress TRH. Since these data are only preliminary, additional experiments are in progress to better define these actions and to determine the primary site(s) of action of PFDA in producing these effects.

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The Effects of Perfluoro-*n*-decanoic Acid in the Rat Heart[1]

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The Effects of Perfluoro-*n*-decanoic Acid in the Rat Heart. PILCHER, G. D., AND LANGLEY, A. E. (1986). *Toxicol. Appl. Pharmacol.* **85**, 389–397. Perfluoro-*n*-decanoic acid (PFDA) is a synthetic chemical resembling a 10-carbon fatty acid. Several studies have suggested that the toxic mechanism of PFDA may involve impaired lipid metabolism and/or altered cell membrane function. We examined the possibility that altered cell membrane structure in the heart might lead to changes in the functional activity of the organ. Functional characteristics were determined in the isolated perfused rat heart by measuring the ability of the heart to respond to either sympathetic nerve stimulation or infused norepinephrine. PFDA reduced the intrinsic resting heart rate and the inotropic response to a stimulus with maximal effects occurring 8 days after dosing. In addition, resting heart rate measured *in vivo* was found to be reduced in PFDA-treated rats 6 to 8 days after dosing. β-Receptor binding studies conducted 8 days after a single dose of PFDA showed that the maximum binding capacity was reduced by PFDA treatment without significant changes in receptor affinity. It is concluded that the reduction in the inotropic response to catecholamines following PFDA treatment may be explained in part by lower β-receptor density in the myocardial cell membrane. These effects may be related to the early fall in serum thyroid hormone levels as previously reported.

Perfluoro-*n*-decanoic acid (PFDA; nonadecafluorodecanoic acid, $C_{10}F_{19}O_2H$) is a straight-chain 10-carbon carboxylic acid with fluorine substituted for all hydrogens at the C-2 through C-10 carbon atoms. Straight-chain perfluorocarboxylic acids 8 to 12 carbons in length and structurally similar perfluorinated derivatives have a broad range of commercial application. They are used in electroplating and in imparting water and oil resistance to fabrics, leather, and food wrapping paper (Bryce, 1964). In addition, the surfactant nature of perfluorinated fatty acyl compounds allows them to be used in the aqueous polymerization of fluorinated monomers (Griffith and Long, 1980) and in aqueous film-forming foams used in fire extinguishant mixtures (Shinoda and Nomura, 1980).

Many perfluorinated compounds are chemically inert (Clarke *et al.*, 1973) and some are reported to be persistently retained in experimental animals for significant time periods following exposure (Clarke *et al.*, 1970). In addition, certain perfluorinated carboxylic acids have been found in the serum of fluorochemical workers long after exposure had been discontinued (Ubel *et al.*, 1980). In that study, the authors noted that no adverse health effects were apparent among the workers though the persistent nature of these chemicals might result in long-term toxicity.

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The acute toxicity of PFDA includes hypophagia with severe weight loss and delayed lethality. Olson and Andersen (1983) reported an LD50 in male Fisher 344 rats of 64 mg/kg. Total hepatic fatty acid composition, as well as altered organ weights including a reduction in heart weight, was observed following PFDA exposure (Olson and Andersen, 1983). We have previously reported that PFDA reduces resting heart rate, body temperature, and serum thyroid hormones in rats over a delayed time course (Langley and Pilcher, 1985). The thyroid state can modulate heart mass (Ciaraldi and Marinetti, 1977) as well as cardiac adrenergic function (Gross and Lues, 1985). Based on reported observations that PFDA lowered resting heart rate *in vivo*, reduced heart weight, and reduced serum thyroid hormones in rats, studies were initiated to further define PFDA's effects on cardiac function. In the present work, the effect of PFDA on the functional responses of the isolated perfused rat heart to adrenergic stimuli was investigated. The functional parameters of heart rate (HR) and right ventricular pressure (RVP) were measured in response to (1) sympathetic nerve stimulation or (2) the direct infusion of norepinephrine (NE). In addition, the effect of PFDA on the binding characteristics of myocardial β-receptors was investigated.

METHODS

Materials. PFDA (98%) was obtained in crystalline form from Aldrich Chemical Company (Milwaukee, Wisc.). The salts used in the Krebs–Hensleit perfusate were purchased from Fisher Scientific, Inc. (Cincinnati, Ohio). Propylene glycol, bovine serum albumin, l-norepinephrine HCl, l-isoproterenol HCl, and *dl*-propranolol HCl were obtained from Sigma Chemical Company (St. Louis, Mo.). (−)-[^{3}H]Dihydroalprenolol ([^{3}H]DHA), sp act 90 Ci/mmol, was purchased from New England Nuclear (Boston, Mass.).

Animals. Male Wistar rats (175–225 g) from Harlan Sprague–Dawley, Inc. (Indianapolis, Ind.) were housed separately in a temperature-controlled room with an 11-hr/13-hr light–dark cycle. The rats were fed standard lab chow (Ralston–Purina Formulab 5508) and given tap water *ad libitum* for a period of 1 week prior to use. Dosing solution (75 mg PFDA/ml) was prepared in a propylene glycol–water vehicle (1:1) and administered in a volume of 1 ml/kg body wt. For the initial time-course study, groups of four to six rats were given a single dose of PFDA (75 mg/kg) between 1600 and 1700 hr, then sacrificed by decapitation 4, 6, 8, or 10 days after dosing for isolated heart experiments with sympathetic nerve stimulation. Serum was collected from these animals for thyroid hormone determinations. Body weights and food consumption were measured daily to the nearest gram. Weight-matched, pair-fed controls were injected with vehicle (1 ml/kg) and given the amount of food consumed daily by the corresponding PFDA-treated rats. Pair-fed controls were sacrificed 4, 6, 8, or 10 days after injection with vehicle; hearts were isolated and sera collected for thyroid hormone determinations. In addition, hearts were isolated and sera collected from a group of control rats allowed to feed *ad libitum*. Data on serum thyroid hormone levels were previously reported (Langley and Pilcher, 1985).

In the norepinephrine infusion experiment, hearts were isolated from six PFDA, six pair-fed, or six control *ad lib*-fed rats 8 days after dosing with PFDA (75 mg/kg) or vehicle (1 ml/kg).

Isolated, perfused heart experiments. Hearts were prepared using a modification of the Langendorff procedure (Langley and Weiner, 1980). Following decapitation, hearts were rapidly exposed by removal of the sternum and perfused *in situ* via the ascending aorta at 7 ml/min with a modified Krebs/Henseleit solution containing 118 mM NaCl, 27.2 mM $NaHCO_3$, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.8 mM KCl, 0.5 mM EDTA, 11.1 mM Dextrose, 2.5 mM $CaCl_2$, and 0.4% w/v BSA. The oxygen tension and pH of the perfusion solution were maintained by bubbling 95% O_2/5% CO_2 through the solution. The temperature of the perfusate entering the heart was maintained at 32 ± 0.3°C by an MGW Lauda Model T-1 constant temperature bath.

In experiments which involved nerve stimulation, both right and left stellate ganglia with intact sympathetic postganglionic nerve fibers to the heart were carefully isolated and dissected away from the surrounding tissue. The heart with intact sympathetic innervation was removed from the thorax and suspended via the aortic cannula. A PE-200 catheter connected to a Statham P-23 D pressure transducer was inserted into the right ventricle via the pulmonary trunk for RVP measurements. In addition, a three-lead electrogram recording with integrated HR was obtained from three platinum electrodes placed on the surface of the isolated heart and connected to a Gould Ecg-Biotach amplifier unit. RVP, HR, and the electrograms were simultaneously recorded using a four-channel Gould 2400S physiological recorder. The postganglionic nerve bundles were carefully suspended from the bipolar stimulation electrodes of a Grass Model SD-9 stimulator. Stimulation of the nerves was applied with supramaximal voltage (approximately 10 V), a 2.0-msec impulse duration, and a 0.02-msec delay. Stimulations were applied at various frequencies (0.2, 0.4, 0.8,

1.0, 2.0, 4.0, and 8.0 Hz) and stimulation periods (5.0, 2.5, 1.25, 1.0, 1.0, and 1.0 min, respectively), with a 10-min interval between each stimulation period.

In isolated heart experiments involving the direct infusion of NE hearts were perfused and suspended from the aortic cannula as described above without prior isolation of the sympathetic nerve fibers. Infusions were carried out using a 21-gauge 1½-in. syringe needle connected by PE-160 tubing to a 5-ml syringe mounted in a Harvard Apparatus compact infusion pump which maintained a constant rate of drug infusion. Varying concentrations of NE were delivered to the heart by placing the syringe needle into the rubber tubing of the perfusion system near the aortic cannula and varying the infusion rate of a 5×10^{-5} M working solution of NE from the syringe pump. The working solution of NE was prepared prior to each experiment from a stock solution of NE (1×10^{-2} M) stabilized with 0.2 mM sodium metabisulfite. The flow of the syringe pump was less than or equal to 1% of the total perfusion flow rate. There were 10- to 15-min "washout" periods between each infusion period. Recordings of RVP, HR, and the electrograms were obtained as described above.

Prestimulation parameters were recorded immediately prior to each stimulation or infusion period. The maximum-stimulated values were the peak values of RVP or HR noted during the stimulation or infusion period.

Cardiac membrane preparation (30,000g fraction). The method of Williams *et al.* (1977) was used to prepare "cardiac membrane fragments" for [^{3}H]DHA binding. Pooled hearts were minced with scissors and homogenized in 10 vol of ice-cold buffer (0.25 M sucrose, 5 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$), using a Brinkman Polytron PCU-2 homogenizer at one-half maximal speed for 15 sec. The homogenate was centrifuged at 480*g* for 10 min at 4°C to remove cellular debris. The resulting supernatant was centrifuged at 30,000*g* for 10 min at 4°C. The resulting pellet was washed by resuspending in incubation buffer (50 mM Tris–HCl, pH 7.5, and 10 mM $MgCl_2$) and recentrifugation at 30,000*g* for 10 min at 4°C. The final pellet was resuspended in incubation buffer to yield a protein concentration of 3 to 5 mg/ml. Protein content was determined by the method of Lowry *et al.* (1951). All centrifugation steps were performed in a Dupont Sorvall RC-5 Superspeed refrigerated centrifuge.

[^{3}H]Dihydroalprenolol binding. Saturation binding experiments were carried out with aliquots from the resuspended 30,000*g* pellet. The aliquots were incubated at 37°C for 10 min in a volume of 200 μl containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, (−)-[^{3}H]DHA (0.1 to 4 nM) and 100 to 200 μg of membrane protein. Duplicate samples were run at each concentration of [^{3}H]DHA in the presence and absence of 10 μM *dl*-propranolol. The reaction was terminated by addition of 2 ml of ice-cold incubation buffer to each sample followed by rapid vacuum filtration through Whatman GF/C filters. The filters were washed with an additional 10 ml of cold incubation buffer and allowed to dry. Filters were placed in scintillation vials containing 4 ml of Aquasure liquid scintillation cocktail (New England Nuclear, Boston, Mass.) and counted in a Packard PL Tri-Carb liquid scintillation counter. Specific binding to β-adrenergic receptors was determined by subtracting the [^{3}H]DHA bound in the presence of 10 μM propranolol (nonspecific binding) from that in the absence of propranolol (total binding). Specific binding was approximately 60–80% of total [^{3}H]DHA binding. The amount of [^{3}H]DHA bound was calculated from the specific activity of the [^{3}H]DHA and expressed as femtomoles [^{3}H]DHA bound per milligram protein. The saturation binding data were analyzed by the method of Scatchard (1949).

Competition binding experiments with adrenergic agonists. In order to investigate β-adrenoceptor agonist binding characteristics, a protocol similar to that used by Kent *et al.* (1980) was employed. Aliquots of the cardiac membrane preparation (100 to 200 μg protein) were incubated in a volume of 200 μl containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 2 nM (−)-[^{3}H]DHA, and varying concentrations of either isoproterenol (5 nM to 50 μM) or norepinephrine (50 nM to 500 μM). Incubations were for 10 min at 37°C. The reaction was terminated with cold buffer and filtered through Whatman GF/C filters. Filters were counted as described above. Agonist binding was quantitated by determining the percentage displacement of bound [^{3}H]DHA at various concentrations of agonist. The percentage agonist bound versus agonist concentration was analyzed with a nonlinear least-squares curve-fitting procedure (Statistical Analysis Systems Institute, Cary, N.C.), using the general model for ligand–receptor binding described by Birdsall *et al.* (1980). The experimental binding data were iteratively fit using the model for one, two, or three classes of binding sites. The model yielding the lowest value of the mean squares of residuals provided the best fit for the data. The computer analysis provided estimates of the affinity state(s) and relative proportion of each binding site.

RESULTS

The Effects of PFDA on Cardiac Function

Isolated rat heart with sympathetic nerve stimulation. A time course for cardiac effects of PFDA was established by isolating hearts 4, 6, 8, or 10 days after treatment. Stimulation of the sympathetic nerve bundles resulted in an increase in both HR and RVP in all treatment groups. The nerve-stimulated increase in heart rate (ΔHR) was enhanced in

TABLE 1

EFFECT OF SYMPATHETIC NERVE STIMULATION ON HEART RATE (BEATS/MIN) IN THE ISOLATED RAT HEART

Days after treatment		Stimulation frequency (Hz)						
		0.2	0.4	0.8	1.0	2.0	4.0	8.0
		Ad libitum control						
$n = 5$	S[a]	247 ± 3	262 ± 4	291 ± 12	291 ± 10	317 ± 15	342 ± 11	343 ± 15
	P[b]	232 ± 3	228 ± 6	235 ± 5	231 ± 5	227 ± 6	236 ± 6	245 ± 4
	Δ	16 ± 6	34 ± 7	56 ± 13	60 ± 13	89 ± 14	106 ± 12	98 ± 13
		PFDA						
4	S	257 ± 13	283 ± 12[c]	276 ± 23	275 ± 25	302 ± 17[c]	318 ± 12[c]	322 ± 17
($n = 4$)	P	204 ± 8	224 ± 11	214 ± 11	209 ± 15	206 ± 7	207 ± 5	213 ± 10
	Δ	53 ± 6	59 ± 7[c]	62 ± 18	64 ± 13	97 ± 11[c]	112 ± 8[c]	109 ± 9
6	S	259 ± 23	259 ± 16	272 ± 21	279 ± 19	311 ± 19	330 ± 15	322 ± 17
($n = 6$)	P	205 ± 8	200 ± 7[d]	191 ± 6[d]	194 ± 7	192 ± 6[d]	191 ± 7[d]	191 ± 7[d]
	Δ	54 ± 20	59 ± 16	82 ± 19	85 ± 18	119 ± 18	139 ± 17[c]	113 ± 19
8	S	202 ± 13[d]	241 ± 16	257 ± 19	263 ± 19	287 ± 23	301 ± 23	315 ± 19
($n = 6$)	P	169 ± 10[d]	178 ± 6[d]	187 ± 16	181 ± 15[d]	169 ± 7[d]	179 ± 11[d]	171 ± 7[d]
	Δ	33 ± 7	63 ± 11[c]	70 ± 14	82 ± 14[c]	119 ± 16[c]	125 ± 16	144 ± 13[c]
10	S	222 ± 13	236 ± 30	249 ± 26	255 ± 25	292 ± 16	305 ± 23	322 ± 23
($n = 3$)	P	185 ± 6	181 ± 16	174 ± 10	172 ± 11	177 ± 9	172 ± 6	183 ± 6
	Δ	37 ± 9	55 ± 17	75 ± 21	83 ± 24	115 ± 23	133 ± 28	139 ± 27
		Pair-fed control						
4	S	238 ± 6	234 ± 4	250 ± 6	244 ± 2	255 ± 7	265 ± 12	292 ± 15
($n = 4$)	P	207 ± 5	218 ± 5	212 ± 6	206 ± 7	210 ± 9	207 ± 10	209 ± 7
	Δ	31 ± 6	16 ± 6	37 ± 7	39 ± 8	46 ± 14	58 ± 15	83 ± 21
6	S	237 ± 12	242 ± 10	257 ± 16	266 ± 15	289 ± 15	309 ± 13	316 ± 14
($n = 6$)	P	229 ± 10	218 ± 9	217 ± 10	222 ± 9	220 ± 10	222 ± 12	232 ± 10
	Δ	8 ± 3	25 ± 9	41 ± 8	44 ± 10	69 ± 15	87 ± 9	87 ± 11
8	S	250 ± 11	258 ± 11	262 ± 10	265 ± 9	280 ± 10	306 ± 9	309 ± 12
($n = 6$)	P	226 ± 8	226 ± 9	223 ± 10	226 ± 12	223 ± 10	219 ± 8	222 ± 12
	Δ	24 ± 3	32 ± 5	40 ± 7	39 ± 5	57 ± 9	87 ± 9	87 ± 15
10	S	245 ± 14	258 ± 25	275 ± 47	253 ± 17	282 ± 3	310 ± 0	312 ± 6
($n = 3$)	P	223 ± 24	232 ± 12	225 ± 29	203 ± 28	210 ± 21	193 ± 30	203 ± 7
	Δ	22 ± 18	26 ± 18	50 ± 22	49 ± 21	72 ± 18	117 ± 30	109 ± 12

Note. Values are means ± SE.

[a] S = the maximum HR attained during the stimulation period.

[b] P = the resting (prestimulation) HR recorded immediately prior to the stimulation period.

[c] Significantly greater than pair-fed control, $p < 0.05$, Student's t test.

[d] Significantly less than pair-fed control, $p < 0.05$, Student's t test.

hearts from PFDA-treated rats. The increases in ΔHR were significant at 4 days (0.4, 2.0, and 4.0 Hz), at 6 days (4.0 Hz), and at 8 days (0.4, 1.0, 2.0, and 8.0 Hz). These changes were due primarily to a significant decline in the intrinsic resting HR (Table 1). The greatest reduction in resting HR was found 8 days after PFDA treatment. The maximum nerve-stimulated HRs were enhanced by PFDA only on Day 4 postexposure. The prestimulation measurements of HR did not appear to change significantly during the course of individual experiments or within any treatment group.

The greatest differences in nerve-stimulated right ventricular pressure, ΔRVP, occurred 8 days after a single dose of PFDA (Table 2). Treatment with PFDA resulted in

significantly lower ΔRVP at the higher frequencies of stimulation (2.0, 4.0, and 8.0 Hz), making the ΔRVP response curve very shallow compared to controls. The ΔRVP at 8 Hz was 52% less in hearts isolated from PFDA-treated rats compared to hearts isolated from pair-fed control rats. PFDA's effect on ΔRVP at 8 days postexposure was due to a decline in the maximum nerve-stimulated RVP rather than a change in prestimulation values of RVP. In addition, at 8 days postexposure, the prestimulation RVP values were stable throughout the experimental period.

The Effects of PFDA on the Responses of the Isolated Rat Heart to Infused Norepinephrine

PFDA's effect on nerve-stimulated responses in the isolated rat hearts was greatest 8 days after treatment. Therefore, a response curve of isolated hearts to infusion of the adrenergic neurotransmitter norepinephrine was generated 8 days after a single dose of PFDA. Again, PFDA caused a significant reduction in resting (intrinsic) HR in the isolated heart preparation without affecting the maximum HR attained at each concentration of norepinephrine (Table 3). As a consequence the ΔHR was enhanced by PFDA treatment especially at the higher concentrations of infused norepinephrine (5, 10, 25, and 50 μM). PFDA's effect on the RVP response to infused norepinephrine was similar to that seen in the nerve-stimulated isolated heart preparation (Table 2). The ΔRVP response to norepinephrine was reduced in the PFDA-treated group at the higher concentrations of norepinephrine (5, 10, 25, 50 μM). Again this effect was due to a reduction in the maximum RVP attained at nearly all concentrations of infused norepinephrine. The prestimulation RVP remained fairly stable throughout the experimental period in hearts from all three groups.

The Effect of PFDA on β-Adrenergic Receptor Binding Characteristics

A Scatchard plot of the saturation binding data (Fig. 1) indicates that PFDA lowered the maximum binding capacity (B_{max}) without changing the affinity of the receptor for [^{3}H]DHA. The observed shift in B_{max} in the PFDA-treated group was statistically significant compared to *ad lib* controls. The mean B_{max} values in fmol [^{3}H]DHA bound/mg protein (± SE) were PFDA, 139.2 ± 27.1; pair-fed control, 178.0 ± 40.6; and control *ad lib*, 206.6 ± 16.4. The affinity of [^{3}H]DHA for binding sites was the same in all three groups. The mean dissociation constants (K_d) (in nM SE) were PFDA, 2.86 ± 0.50; pair-fed control, 2.48 ± 0.45; and control *ad lib*, 2.45 ± 0.39. These were similar to previously reported values (Williams and Lefkowitz, 1978; Stiles and Lefkowitz, 1981; Winek and Bhalla, 1979).

In order to evaluate PFDA's effect on β-receptor agonist binding properties, the kinetics of agonist displacement of a single concentration of bound [^{3}H]DHA (2 nM) was measured is the same cardiac membrane preparation. Either norepinephrine or isoproterenol was tested for its ability to displace [^{3}H]DHA. In each case, a two-site binding model provided the best mathematical estimates for the affinity states of the β-receptor (Table 4). Others have reported that a two-site model provides the optimum fit for β-receptor agonist binding in the rat heart (Kent *et al.*, 1980). PFDA did not alter either the affinity constants (K_d's) or the relative proportion of sites for either norepinephrine or isoproterenol binding. The observed kinetic parameters were similar to those reported by Hancock *et al.* (1979) and Stiles and Lefkowitz (1981) in rat heart.

DISCUSSION

We have previously reported a significant fall in total circulating thyroid hormone levels in sera from the rats used for the isolated heart experiments reported herein (Langley and Pilcher, 1985). In addition to decreases in serum thyroid hormone levels, we observed hypothermia and bradycardia which are common manifestations of hypothyroidism

TABLE 2

EFFECT OF SYMPATHETIC NERVE STIMULATION ON RIGHT VENTRICULAR PRESSURE (mm Hg) IN THE ISOLATED RAT HEART

Days after treatment		Stimulation frequency (Hz) 0.2	0.4	0.8	1.0	2.0	4.0	8.0
				Ad libitum control				
$n = 5$	S[a]	37 ± 2	38 ± 1	41 ± 3	39 ± 2	43 ± 3	46 ± 3	47 ± 3
	P[b]	34 ± 1	32 ± 2	31 ± 2	30 ± 3	28 ± 2	27 ± 2	27 ± 2
	Δ	3 ± 1	5 ± 1	10 ± 1	9 ± 2	15 ± 2	19 ± 1	20 ± 1
				PFDA				
4	S	28 ± 3	29 ± 5	31 ± 7	31 ± 7	36 ± 8	40 ± 7	42 ± 8
($n = 4$)	P	26 ± 3	20 ± 2	20 ± 2	20 ± 2	20 ± 2	18 ± 3	17 ± 3[c]
	Δ	2 ± 2	9 ± 3	11 ± 5	11 ± 5	17 ± 5	22 ± 6	25 ± 7
6	S	36 ± 2	42 ± 2	43 ± 2	43 ± 2	46 ± 2	49 ± 4	46 ± 3[c]
($n = 6$)	P	29 ± 3	31 ± 3	31 ± 2	29 ± 2	30 ± 3	31 ± 2	31 ± 3
	Δ	8 ± 2	11 ± 2	13 ± 2	15 ± 2	16 ± 2	18 ± 4	17 ± 4
8	S	30 ± 3	31 ± 3	33 ± 3	33 ± 3	34 ± 3	34 ± 3	36 ± 4
($n = 6$)	P	26 ± 3	28 ± 3	29 ± 3	28 ± 3	27 ± 3	27 ± 3	26 ± 3
	Δ	4 ± 1	4 ± 1	5 ± 1	6 ± 2	7 ± 2[c]	7 ± 2[c]	10 ± 3[c]
10	S	29 ± 4	32 ± 2	34 ± 3	35 ± 3	37 ± 3	35 ± 3	35 ± 2[c]
($n = 3$)	P	25 ± 4	26 ± 3	24 ± 4	24 ± 4	23 ± 4	23 ± 4	20 ± 2
	Δ	4 ± 1	8 ± 0	10 ± 1	11 ± 1	14 ± 1	12 ± 2	15 ± 1
				Pair-fed control				
4	S	36 ± 6	37 ± 7	41 ± 8	43 ± 8	43 ± 8	48 ± 8	51 ± 8
($n = 4$)	P	29 ± 4	28 ± 4	26 ± 3	25 ± 2	24 ± 2	23 ± 2	24 ± 2
	Δ	7 ± 2	09 ± 2	15 ± 4	18 ± 5	19 ± 5	25 ± 6	27 ± 6
6	S	36 ± 2	37 ± 2	41 ± 2	43 ± 2	45 ± 2	52 ± 2	56 ± 2
($n = 6$)	P	31 ± 3	32 ± 1	31 ± 1	29 ± 2	31 ± 1	27 ± 2	27 ± 2
	Δ	5 ± 2	5 ± 1	10 ± 2	14 ± 2	15 ± 2	23 ± 3	29 ± 2
8	S	35 ± 3	36 ± 2	38 ± 4	38 ± 3	41 ± 4	44 ± 4	47 ± 4
($n = 6$)	P	32 ± 3	32 ± 3	30 ± 3	28 ± 3	27 ± 4	27 ± 4	26 ± 3
	Δ	4 ± 1	4 ± 1	8 ± 1	9 ± 1	14 ± 2	17 ± 2	21 ± 2
10	S	32 ± 3	34 ± 4	38 ± 3	38 ± 4	42 ± 2	44 ± 2	45 ± 2
($n = 3$)	P	28 ± 3	29 ± 4	29 ± 3	28 ± 4	29 ± 3	29 ± 2	28 ± 3
	Δ	4 ± 1	5 ± 1	9 ± 1	10 ± 1	13 ± 3	15 ± 3	17 ± 4

Note. Values are means ± SE.

[a] S = the maximum RVP attained during the stimulation period.

[b] P = the RVP recorded immediately prior to the stimulation period.

[c] Significantly less than pair-fed control, $p < 0.05$, Student's t test.

(Ibbertson, 1979; Mazzaferri, 1980). Hypothyroidism has been shown to lower the number of β-receptors in rat hearts (McConnaughey *et al.*, 1979; Stiles and Lefkowitz, 1981) without changing the binding affinity of the receptor (Stiles and Lefkowitzk, 1981). In addition, the modulation of myocardial β-receptors by thyroid hormone is reflected in altered responsivity of the tissue (Brodde *et al.*, 1980).

An examination of the time course of myocardial effects of a single dose of PFDA showed that maximal changes in sympathetic nerve-stimulated HR and RVP occurred 8 days after treatment with PFDA. The effects of PFDA on the responses of the isolated heart were qualitatively similar when either sympathetic nerve stimulation or infusion of norepinephrine was employed to stimulate cardiac activity. These results indicate that

TABLE 3

EFFECT OF INFUSED NOREPINEPHRINE ON HEART RATE (BEATS/MIN) AND RIGHT VENTRICULAR PRESSURE (mm Hg) IN THE ISOLATED RAT HEART

Treatment group		Norepinephrine ($\times 10^{-7}$ M)						
		0.05	0.10	0.25	0.5	1.0	2.5	5.0
				RVP				
Control	S[a]	39 ± 2	51 ± 6	49 ± 7	59 ± 4	62 ± 3	62 ± 5	59 ± 5
(n = 5)	P[b]	37 ± 1	35 ± 2	29 ± 2	26 ± 2	25 ± 3	20 ± 4	29 ± 7
	Δ	2 ± 2	16 ± 4	20 ± 5	35 ± 2	37 ± 3	42 ± 6	35 ± 7
PFDA	S	35 ± 3	45 ± 3[d]	49 ± 2[d]	50 ± 2[d]	48 ± 3[d]	46 ± 3[d]	46 ± 3[d]
(n = 6)	P	26 ± 2[d]	33 ± 1[d]	34 ± 1	33 ± 2	29 ± 2	26 ± 2	25 ± 3
	Δ	8 ± 3	12 ± 3	15 ± 2	17 ± 2[d]	19 ± 2[d]	21 ± 2[d]	21 ± 3[d]
Pair-fed control	S	45 ± 4	55 ± 1	57 ± 2	60 ± 3	63 ± 3	62 ± 4	62 ± 3
(n = 6)	P	33 ± 3	40 ± 2	39 ± 3	36 ± 3	36 ± 2	31 ± 3	29 ± 3
	Δ	11 ± 3	16 ± 3	18 ± 2	24 ± 2	27 ± 2	32 ± 2	33 ± 2
				HR				
Control	S	227 ± 4	241 ± 8	240 ± 5	253 ± 6	270 ± 12	274 ± 15	291 ± 21
(n = 5)	P	223 ± 4	226 ± 8	228 ± 8	224 ± 5	224 ± 5	227 ± 5	224 ± 7
	Δ	4 ± 1	15 ± 8	12 ± 6	28 ± 7	46 ± 10	47 ± 13	67 ± 18
PFDA	S	190 ± 7	200 ± 11	194 ± 8	207 ± 16	225 ± 14	241 ± 12	250 ± 10
(n = 6)	P	184 ± 8[d]	190 ± 11	172 ± 8	170 ± 7[d]	168 ± 6[d]	167 ± 9[d]	161 ± 8[d]
	Δ	6 ± 2	11 ± 3	22 ± 5	37 ± 17	57 ± 13	74 ± 9[c]	89 ± 9[c]
Pair-fed control	S	221 ± 7	215 ± 6	211 ± 6	212 ± 8	221 ± 8	224 ± 8	234 ± 6
(n = 6)	P	215 ± 4	196 ± 7	189 ± 6	197 ± 7	195 ± 6	201 ± 4	197 ± 7
	Δ	6 ± 3	18 ± 6	22 ± 7	15 ± 9	27 ± 6	23 ± 7	37 ± 9

Note. Values are means ± SE.

[a] S = the maximum HR or RVP attained during the infusion period.

[b] P = the HR or RVP recorded immediately prior to the infusion period.

[c] Significantly greater than pair-fed control, $p < 0.05$, Student's t test.

[d] Significantly less than pair-fed control, $p < 0.05$, Sudent's t test.

the effects are apparently mediated by an action on the myocardium rather than on the release of norepinephrine in response to stimulation of the sympathetic nerves.

PFDA caused the ΔHR and ΔRVP elicited by nerve stimulation or norepinephrine infusion in the isolated heart to deviate from control values in opposite directions. It must be emphasized that PFDA's effect on ΔHR was largely due to a reduction in intrinsic (resting) HR with no significant change in maximum HR, whereas the reduced ΔRVP was the result of a lower maximum RVP. This effect of PFDA on resting HR *in vitro* is similar to the effect on resting HR *in vivo* previously reported (Langley and Pilcher, 1985). The fact that PFDA reduced resting HR *in vitro* as well as *in vivo* suggests that PFDA produces this effect by an action on the myocardium rather than via the autonomic nerves since the isolated heart lacks autonomic influences in the resting situation. Since diastolic depolarization in dominant pacemaker cells of the sinoatrial (SA) node normally controls HR (Vassalle, 1982), our data suggest a PFDA-induced alteration in SA nodal function.

The effect of PFDA on the inotropic response of the isolated heart to norepinephrine was manifested in a lower ΔRVP due to a reduction in the maximum stimulated RVP. This reduction was most pronounced at higher rates of nerve stimulation or at higher infused concentrations of norepinephrine, suggesting that the heart's ability to respond

maximally was impaired by PFDA treatment.

The mechanism of enhancement of contractility by catecholamines involves a sequence of events initiated by stimulation of the β-adrenergic receptor on the surface of the myocardial cell which ultimately leads to an enhancement of intracellular Ca^{2+} availability (Katz, 1977). Responsiveness to catecholamines can be modulated at the membrane-receptor level by either a change in the number of receptors and/or an alteration of the affinity of the receptor for an agonist. PFDA reduced the apparent number of β-receptors without a significant change in the affinity of the receptor which may account for the decreased inotropic response observed with either nerve stimulation or NE infusion. The decrease in *in vitro* HR, reduced inotropic response of the heart to stimulation, and reduced number of β-receptor binding sites reported herein may reflect the changes that we observed in serum thyroid hormone levels. These data tend to support our previous conclusion that some of the biological changes that occur several days after PFDA treatment may in part be secondary to early decreases in serum thyroid hormone levels (Langley and Pilcher, 1985).

TABLE 4

BINDING PARAMETERS OF β-ADRENERGIC RECEPTOR AGONISTS ESTIMATED BY COMPETITIVE ANTAGONISM OF [^{3}H]DHA BINDING

Treatment group	Site	Affinity constant (K_a)[a]	Relative proportion of site
		Norepinephrine	
Control (*ad lib*)	A	$6.66 \pm 1.30 \times 10^3$	0.56
	B	$7.32 \pm 1.91 \times 10^5$	0.44
PFDA	A	$7.71 \pm 2.02 \times 10^3$	0.50
	B	$1.50 \pm 0.41 \times 10^6$	0.50
Pair-fed control	A	$3.56 \pm 1.25 \times 10^3$	0.43
	B	$9.86 \pm 2.49 \times 10^5$	0.57
		Isoproterenol	
Control (*ad lib*)	A	$5.58 \pm 1.33 \times 10^5$	0.55
	B	$1.22 \pm 0.65 \times 10^7$	0.45
PFDA	A	$3.49 \pm 0.90 \times 10^5$	0.56
	B	$3.23 \pm 0.99 \times 10^7$	0.44
Pair-fed control	A	$3.25 \pm 0.76 \times 10^5$	0.47
	B	$2.92 \pm 0.55 \times 10^7$	0.53

[a] The estimates of K_a are expressed as means ± asymptotic standard error.

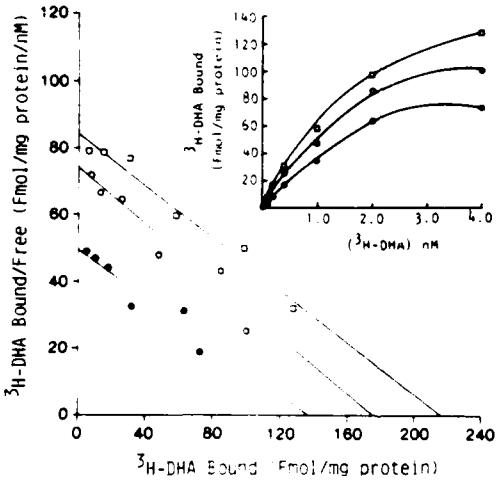

FIG. 1. Saturation binding isotherm with Scatchard plot of [^{3}H]DHA binding in cardiac membrane fragments from PFDA (●, $n = 4$), pair-fed (○, $n = 4$), and control *ad lib* (□, $n = 4$) rats 8 days postexposure. Saturation binding was determined over a range of [^{3}H]DHA concentrations from 0.1 to 4 nM. The lines for PFDA ($r = 0.98$), pair-fed control ($r = 0.95$), and control *ad lib* ($r = 0.98$) in the Scathchard plot were determined by linear regression analysis. Estimates of the dissociation constant (K_d) and maximum binding capacity (B_{max}) for each experiment were obtained from $-1/$slope and the x intercept, respectively. Differences between PFDA-treated and control groups were determined using Student's t test, $p < 0.05$.

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The Effects of Perfluoro-*n*-decanoic Acid (PFDA) on Rat Heart β-Receptors, Adenylate Cyclase, and Fatty Acid Composition[1]

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The Effects of Perfluoro-*n*-decanoic Acid (PFDA) on Rat Heart β-Receptors, Adenylate Cyclase, and Fatty Acid Composition. PILCHER, G. D., GUTSHALL, D. M., AND LANGLEY, A. E. (1987). *Toxicol. Appl. Pharmacol.* **90**, 198–205. Perfluoro-*n*-decanoic acid (PFDA) is a member of a family of surfactants with numerous industrial applications. The acute toxicity of PFDA is characterized by body wasting and delayed lethality. Recent reports have indicated that the effects of PFDA may involve an action on the structure of biological membranes which results in an alteration of function. In the present study we extend our work on the membrane actions of PFDA by examining its effects on myocardial β-adrenoceptor binding characteristics and adenylate cyclase. Following a single injection of PFDA the apparent number of β-receptor binding sites was reduced compared to pair-fed controls. This change in β-receptor binding capacity was reflected in a reduced ability of norepinephrine to activate adenylate cyclase. No alterations were observed in basal adenylate cyclase activity or in the ability of NaF or guanylyl imidodiphosphate to stimulate the enzyme. The fatty acid composition of the heart was changed by PFDA treatment. Our results suggest that the toxic effects of PFDA may be due to an alteration of the membrane lipid bilayer leading to changes in the functional activity of myocardial membranes.

Perfluoro-*n*-decanoic acid (PFDA) or nonadecafluorodecanoic acid is a 10 carbon straight-chain fatty acid, $C_{10}F_{19}O_2H$, having complete substitution of fluorine for hydrogen in the aliphatic portion of the molecule. Perfluoroalkanoic acids and structurally related compounds are used widely in manufacturing as anti-wetting agents (Guenthner and Vietor, 1962) and in the treatment of fabric and paper (Bryce, 1964).

In rats a single dose of PFDA near the LD50 has been reported to produce hypophagia and severe weight loss (Olson and Andersen, 1983; Langley and Pilcher, 1985) bradycardia, hypothermia, and decreased circulating levels of thyroid hormones (Langley and Pilcher, 1985). We previously reported reduced responsiveness of isolated rat hearts to adrenergic stimulation and a decrease in the apparent number of β-receptor binding sites in hearts from PFDA-treated rats (Pilcher and Langley, 1986).

Several studies have indicated that PFDA alters lipid metabolism, causing major shifts in hepatic fatty acid composition (Olson and Andersen, 1983; George and Andersen, 1986) as well as tissue cholesterol levels (George and Andersen, 1986) and significant induction of peroxisomal fatty acid oxidation (Van Rafelghem *et al.*, 1985). Other reports have suggested that exposure to PFDA results in altered plasma membrane function either via direct cellular exposure *in vitro* (Rogers *et al.*, 1982; Wigler and Shah, 1986; Levitt and

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Liss, 1986) or in red blood cells following *in vivo* exposure (Olson *et al.*, 1983). In the present study the effect of PFDA on the biochemical transducer of β-adrenergic receptor-mediated activation of the myocardium, adenylate cyclase, was investigated. In addition, β-receptor binding characteristics were examined in the same fraction and correlated with changes in adenylate cyclase. PFDA-induced changes in fatty acid composition of rat myocardium were measured.

METHODS

Male Wistar rats weighing 175–225 g were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN). Rats were housed separately on wood chip bedding in a temperature-controlled room (22.8 ± 2.7°C) with an 11-hr/13-hr light/dark cycle. The rats were fed a standard rat chow, Ralston Purina Formulab No. 5508, and given tap water *ad libitum* until they were used, typically 5–7 days after arrival.

In all experiments, PFDA treatment consisted of ip administration of 75 mg/ml dissolved in a propylene glycol–water (1:1) vehicle delivered in a volume of 1 ml/kg body wt, a dose near the reported LD50 (Olson and Andersen, 1983). Weight-matched pair-fed controls were given the same vehicle in a volume of 1 ml/kg body wt and then pair-fed to the daily food consumption record of the appropriate PFDA-treated animals. In all experiments, rats were given a single dose of PFDA or vehicle and killed 7 or 8 days after injection for collection of heart tissue. No animals died during this interval. *Ad libitum* controls were untreated and allowed to eat and drink *ad libitum*.

β-Receptor binding and adenylate cyclase activity were measured in a crude heart fraction prepared by the method of Drummond and Severson (1974). Rats were decapitated, and their hearts were rapidly removed and chilled in ice-cold homogenizing buffer. The hearts were blotted, weighed, trimmed of excess connective tissue, minced with scissors, and homogenized in 10 vol of fresh homogenizing buffer at 4°C (0.25 M Sucrose, 25 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 0.5 mM EGTA) with a Brinkman Polytron PCU-2 at ½ maximum speed for 20 sec. The crude homogenate was passed through 4-ply cheesecloth to remove additional connective tissue and then centrifuged at 1000*g* for 15 min at 4°C. The resulting pellet was washed twice by resuspending in the original volume of homogenizing buffer and recentrifuged at 1000*g*. The final pellet was resuspended in 10 vol of incubation buffer (25 mM Tris–HCl, pH 7.5, containing 5 mM $MgCl_2$, 2 mM DTT, and 0.5 mM EGTA) yielding a protein concentration of 5 to 10 mg/ml. Proteins were determined by the method of Lowry *et al.* (1951). The protein content of hearts from the different treatment groups was not significantly different (Pilcher, 1985).

[^{3}H]Dihydroalprenolol ([^{3}H]DHA) binding. Saturation binding experiments were carried out with aliquots of the resuspended 1000*g* pellet using [^{3}H]DHA as previously described (Pilcher and Langley, 1986). Specific binding was approximately 50% of total [^{3}H]DHA binding. The saturation binding data were analyzed by the method of Scatchard (1949).

Adenylate cyclase assay. Adenylate cyclase activity was assayed at 30°C using the method of Salomon *et al.* (1974). The incubation mixture contained 25 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM creatine phosphate, 100 units creatine phosphokinase/ml, 1 mM cyclic adenosine monophosphate (cAMP), 1 mM 3-isobutyl-1-methyl-xanthine, 2 mM DTT, 0.5 mM EGTA, 0.02% ascorbic acid, 20 μM guanosine triphosphate (GTP), and 1 mM adenosine triphosphate (ATP) containing at least 1–2 μCi [α-^{32}P]ATP in a total volume of 100 μl. When the nonhydrolyzable GTP analog guanylyl imidodiphosphate (GppNHp) was included in the incubation mixture, 10 μM GppNHp was substituted for GTP. Varying concentrations of norepinephrine or 10 mM NaF were tested for their ability to stimulate adenylate cyclase. The procedure used to calculate enzyme activity was described by Salomon (1979). Enzyme activity was expressed as picomoles cAMP formed per 10 min per milligram protein.

Thermodynamic analysis of basal adenylate cyclase activity. The temperature dependence of adenylate cyclase was examined using the method of Chatelain *et al.* (1982). A 250-μl aliquot of adenylate cyclase incubation medium (described above) was allowed to equilibrate at the appropriate temperature for 1 min and the reaction was initiated by the addition of 50 μl of the heart homogenate. Aliquots of 50 μl were removed from the mixture after 2, 4, 6, 8, and 10 min and rapidly placed in tubes containing 100 μl of 2% sodium dodecyl sulfate, 40 mM ATP, and 1 mM cAMP to stop enzyme activity. Incubations were performed at 15, 20, 24, 26.5, 29, 30, 31.5, 33, 35, and 37°C. At temperatures of 24°C and below, aliquots of the incubation mixture were removed at 2, 5, 10, 15, and 20 min following the addition of heart homogenate. The reactions were found to be linear with time, and a rate constant, *k*, was determined by plotting enzyme activity (pmol cAMP formed/mg protein) against time at each temperature. The rate constants of individual experiments were used for Arrhenius plots (Daniels and Alberty, 1975). To determine discontinuities, Arrhenius plots were analyzed using a two-segment line model as described by Bogartz (1968). Linear regression analyses were performed on each line segment to obtain a total sum of squared deviations for both segments. The two-segment line model which yielded the minimum total sum of squared deviations provided the optimum "fit" for the data. In each treatment group, a two-segment line model provided a significantly better

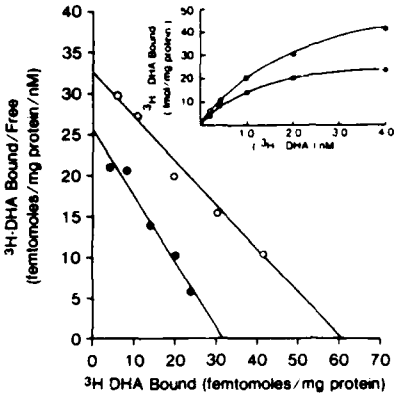

FIG. 1. Saturation binding isotherm with Scatchard plot of [3H]DHA binding from PFDA (●), n = 4, and pair-fed (○), n = 4, rat hearts. Saturation binding was determined over the range of [3H]DHA concentrations from 0.2 to 4 nM. The lines for PFDA (r = 0.98) and pair-fed control (r = 0.99) in the Scatchard plot were determined by linear regression analysis. Differences between PFDA-treated and pair-fed control groups were determined using Student's t test, $p < 0.05$.

"fit" than a single straight line. The energies of activation (E_a's) for adenylate cyclase were derived from the slope of each line segment using the Arrhenius equation.

Analysis of myocardial fatty acid composition. In a separate experiment, rats were injected with PFDA (75 mg/kg) or pair-fed as described above. Rats were killed 7 days after dosing, and hearts were removed, chilled in ice-cold saline, blotted, weighed, and homogenized in 30 ml chloroform:methanol (2:1). Lipids were extracted by the method of Folch *et al.* (1957). The extraction solvents contained 0.05% butylated hydroxytoluene (BHT) to inhibit autooxidation. An aliquot of the total lipid extract was transesterified using BF_3-methanol by the method of Morrison and Smith (1964) to obtain fatty acid methyl esters (FAME). Samples were analyzed on a Varian 3700 gas chromatograph equipped with a 25 m × 0.25 mm i.d. fused silica BP-15 capillary column (Scientific Glass Engineering, Inc.) and flame ionization detector. Analyses were carried out isothermally at 195°C using a split ratio of 78:1. FAME peaks were identified by comparing their relative retention times to those of authentic FAME reference mixtures. A VISTA 402 recording integrator (Varian Associates, Sunnyvale, CA) provided integrated peak areas. Data are expressed as mean area percentage ± SE.

Materials. PFDA (nonadecafluorodecanoic acid, 98%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). (−)-[^{3}H]Dihydroalprenolol, sp act 33 Ci/mmol, and [α-^{32}P]adenosine 5'-triphosphate, sp act 90 Ci/mmol, were purchased from New England Nuclear (Boston, MA). The [2,8-^{3}H]adenosine 3',5'-cyclic monophosphate, sp act 15–30 Ci/mmol, was obtained from ICN radioisotope division (Irvine, CA). Organic solvents were purchased from Fisher Scientific (Cincinnati, OH) and each solvent was distilled in glass prior to use. Boron trifluoride in methanol, fatty acid methyl ester reference mixtures, AOCS rapeseed oil, and polyunsaturated fatty acid No. 2 were purchased from Supelco, Inc. (Bellefonte, PA). All other chemicals were obtained in high purity from Sigma Chemical Co. (St. Louis, MO).

Statistical analyses. Comparisons between the mean values of two treatment groups were made using the Student t test at a significance level of $p < 0.05$. Multiple comparisons between treatment means were analyzed by one-way ANOVA and Duncan's multiple range test at a significance level of $p < 0.05$. Tests were performed using

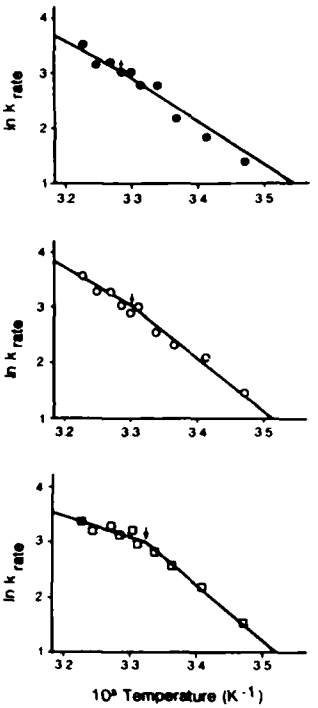

FIG. 2. Arrhenius plots of basal adenylate cyclase activity. Each point represents the mean of two to four determinations. PFDA (●); Pair-fed (○); *ad libitum* control (□). Plots were constructed according to the Arrhenius equation. In each group a two-segment line model provided a significantly better fit than a straight line model. Experiments were analyzed individually to obtain estimates of activation energies above ($E_{act\,1}$) and below the break-point temperature ($E_{act\,2}$). In addition, the break-point temperature (arrow) was determined in individual experiments. Differences between treatment groups were determined using ANOVA, $p < 0.05$, with Duncan's multiple range test on the means.

TABLE 1

ENERGIES OF ACTIVATION (kcal/mol) ABOVE (E_a 1) AND BELOW (E_a 2) THE PHASE TRANSITION TEMPERATURE

	E_a 1	E_a 2
Ad libitum control	−5.02 ± 1.15	−20.46 ± 2.39
Pair-fed control	−13.57 ± 1.51*	−18.91 ± 2.17
PFDA	−12.81 ± 3.11*	−15.50 ± 1.84

* Significantly greater that *ad libitum* control, $p < 0.05$.

SAS (Statistical Analysis Systems, SAS Institute, Cary, NC) on an IBM 370 computer.

RESULTS

Binding of [^{3}H]DHA

A Scatchard plot of the saturation binding of [^{3}H]DHA indicated a decrease in the maximum number of binding sites with no change in affinity (Fig. 1). The mean B_{max} value for the PFDA-treated group (36.0 ± 7.0 fmol/mg) was significantly lower than pair-fed controls (63.0 ± 5.8 fmol/mg) with no significant change in mean K_d values (PFDA, 1.63 ± 0.34 nM; and pair-fed controls, 2.13 ±0.46 nM).

The Effect of PFDA on Adenylate Cyclase Activity

An alteration in the membrane environment of adenylate cyclase might result in changes in the thermodynamic properties of the enzyme or in the ability to activate the enzyme. Adenylate cyclase activity was determined in the 1000*g* fraction. Basal adenylate cyclase activity in preparations from both PFDA and pair-fed controls exhibited discontinuities in their respective Arrhenius plots which are near the reported phase transition temperature of the plasma membrane in rat heart, 31°C (Gordon *et al.*, 1978) (Fig. 2). The discontinuities which were not statistically different were PFDA, 30.9 ± 0.1°C; pair-fed controls, 30.2 ± 0.7°C; and control *ad libitum*, 27.9 ± 1.9°C. Compared with pair-fed controls PFDA did not affect the calculated energies of activation (E_a) either above or below the phase transition point. However, both the PFDA-treated and pair-fed control groups exhibited significantly higher E_a's above the phase transition point compared to controls fed *ad libitum* (Table 1). These data suggest that the reduction in food consumption elevated the E_a for basal adenylate cyclase activity at temperatures above the break-point temperature.

The reduced number of β-receptors following PFDA treatment appeared to be reflected in a decrease in β-receptor-stimulated adenylate cyclase activity. PFDA reduced the ability of norepinephrine (1, 10, and 100 μM) to activate adenylate cyclase (Fig. 3). However, basal activity and activity in the presence of 10 mM sodium fluoride or the nonhydrolyzable guanine nucleotide analog guanylyl imidodiphosphate were not significantly affected by PFDA treatment (Table 2). In addition, the activity of adenylate cyclase in the presence of a high concentration of norepinephrine (1 mM) plus GppNHp was not significantly affected by PFDA treatment (Table 2).

Effect of PFDA on Myocardial Fatty Acid Composition

PFDA treatment caused modest but significant changes in the total fatty acid composition of rat myocardium compared to pair-fed controls (Table 3). Hearts from PFDA-dosed rats had elevated proportions of palmitic (16:0), eicosotrienoic (20:3 ω6), and docosahexaenoic (22:6 ω3) acids of 13, 71, and 18%, respectively. Conversely, the proportion of arachidonic acid (20:4) in hearts from PFDA-dosed rats was reduced 18% below that found in hearts from pair-fed controls.

DISCUSSION

The results of the binding experiments are similar to those previously reported in a

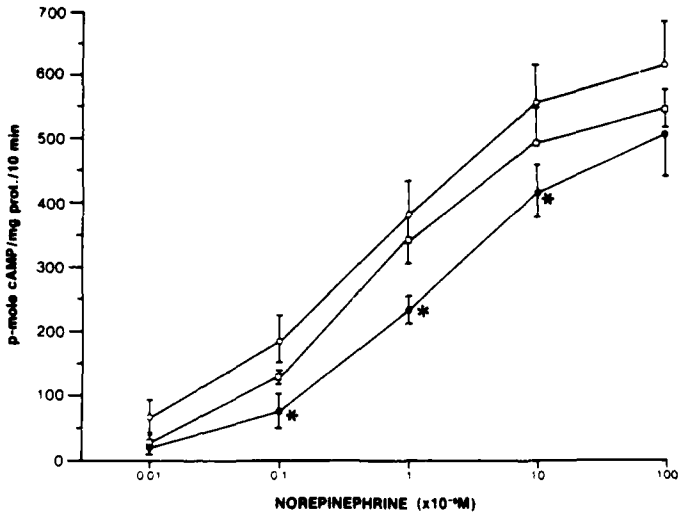

FIG. 3. Norepinephrine-stimulated adenylate cyclase activity. Each point represents the mean ± SE. *Significantly less than pair-fed controls, $p < 0.05$, Student's t test. PFDA (●), $n = 4$; pair-fed controls (○), $n = 4$; *ad libitum* controls (□), $n = 5$.

30,000*g* cardiac preparation (Pilcher and Langley, 1986). PFDA significantly reduced the apparent number of β-receptors without altering the affinity of the receptor. The kinetic constants of dissociation (K_d's) derived by Scatchard analysis of [^{3}H]DHA binding were in agreement with previously reported values (Williams and Lefkowitz, 1978; Stiles and Lefkowitz, 1981; Winek and Bhalla, 1979; Pilcher and Langley, 1986). Wince and Rutledge (1981) reported that alterations of the fatty acid composition of rat atrial membranes through manipulation of dietary lipids reduced β-receptor density and affinity for binding of [^{3}H]DHA. We observed changes in receptor density but not affinity in membrane fragments from whole rat hearts. Although our results are not in complete agreement with those of Wince and Rutledge (1981), the fact remains that altering the lipid composition of different myocardial membranes can change the binding characteristics of the β-receptor.

Stimulation of cardiac β-receptors results in activation of adenylate cyclase and increased cAMP formation (Sutherland *et al.*,

TABLE 2

ADENYLATE CYCLASE ACTIVITY (pmol cAMP FORMED/mg PROTEIN/10 min)

	Basal ($\bar{X}$ ± SE)	10 mM NaF[a] ($\bar{X}$ ± SE)	GppNHp[a,b] ($\bar{X}$ ± SE)	1 mM NE + GppNHp[a,b] ($\bar{X}$ ± SE)
PFDA ($n = 4$)	300 ± 37	1029 ± 117	225 ± 22	870 ± 24
Pair-fed control ($n = 4$)	301 ± 28	1304 ± 155	330 ± 20	912 ± 79
Control *ad libitum* ($n = 5$)	267 ± 18	758 ± 54	314 ± 32	754 ± 45

[a] Activities expressed with basal activity subtracted.
[b] GppNHp, guanylyl imidodiphosphate, 10 μM.

TABLE 3

EFFECT OF PFDA ON FATTY ACID COMPOSITION OF RAT MYOCARDIUM[a]

Fatty acid[b]	PFDA ($n = 5$)	Pair-fed control ($n = 5$)
16:0	13.4 ± 0.4[c]	11.9 ± 0.2
18:0	21.1 ± 0.4	21.8 ± 0.2
18:1	9.5 ± 0.4	9.7 ± 0.4
18:2	16.8 ± 0.5	18.3 ± 0.6
20:3 ω6	0.7 ± 0.1[c]	0.4 ± 0.1
20:4 ω6	15.0 ± 0.5[d]	18.3 ± 0.4
22:4 ω6	0.6 ± 0.1	0.6 ± 0.1
22:5 ω6	0.6 ± 0.1	0.5 ± 0.1
22:5 ω3	2.4 ± 0.1	2.3 ± 0.1
22:6 ω3	19.5 ± 0.6[c]	16.5 ± 0.8

[a] Data shown represent mean relative percentage total fatty acid ± SE.

[b] Fatty acids are designated by carbon chain length (C) followed by number of double bonds (d), C:d. The ω*X* refers to the number of carbon atoms between the last double bond and the terminal methyl group of the fatty acid chain.

[c] Significantly greater than pair-fed control, $p < 0.05$, Student's *t* test.

[d] Significantly less than pair-fed control, $p < 0.05$, Student's *t* test.

1965). A reduction in β-receptor density could lead to a decrease in the activation of adenylate cyclase by catecholamines. The PFDA-induced decrease in β-receptor number was reflected in a reduced ability of norepinephrine to activate adenylate cyclase. The reduction was greatest at 10 μM norepinephrine which is near the reported ED50 value of 5 μM for adenylate cyclase activation (Drummond and Severson, 1974). In the preparation from PFDA-treated rats there were no changes in basal activity or in the apparent phase transition temperature or energies of activation (E_a) for basal adenylate cyclase compared to pair-fed controls. In both PFDA and pair-fed groups an elevation in the E_a for basal adenylate cyclase above the phase transition temperature was observed compared to controls fed *ad libitum*. This effect can be attributed to fasting since PFDA (75 mg/kg) abolished food consumption 6 to 8 days after dosing (Langley and Pilcher, 1985). Fasting has been reported to alter β-receptor-mediated responses in several tissues (Dax *et al.*, 1981; Barney *et al.*, 1983); however, neither study examined the thermodynamic characteristics of adenylate cyclase. Our data may be the first to indicate that fasting influences the thermodynamic behavior of adenylate cyclase. PFDA did not affect NaF- or GppNHp-stimulated adenylate cyclase, suggesting that neither the guanine nucleotide regulatory subunit nor the catalytic subunit of adenylate cyclase which are components of the inner leaflet of the membrane bilayer was affected by PFDA treatment. It appears that the decreased responsiveness of isolated hearts to sympathetic nerve stimulation or norepinephrine infusion that we previously reported (Pilcher and Langley, 1986) may be due to the decreased number of β-receptors and/or an alteration of the coupling of the receptor component to the other subunits of adenylate cyclase.

Since cellular membranes are known to be asymmetrical with regard to both lipid and protein (Houslay and Gordon, 1983; Stubbs and Smith, 1984), our data suggest that the membrane-altering actions of PFDA are apparently restricted to the outer half of the bilayer. The β-receptor (R) component of the adenylate cyclase complex is part of the outer leaflet of the membrane bilayer and appears to interact with the guanine nucleotide binding (G) protein which along with the catalytic unit (*C*) is entirely contained within the inner leaflet (Schramm and Selinger, 1984). Certain fatty acids appear to play an important role as activators of β-receptor-mediated stimulation of adenylate cyclase (Orly and Schramm, 1975). The basal enzyme activity and enzyme activation through stimulation of the G protein (by guanine nucleotides or NaF) are reportedly influenced by changes in the lipid environment of the inner but not the outer leaflet of the membrane bilayer (Houslay and Gordon, 1983). The observation that catecholamine-stimulated adenylate cyclase was reduced by PFDA treatment without a significant effect on basal- or guanine nucleotide/NaF-stimulated activity suggests that the

structural–functional characteristics of the outer but not the inner half of the sarcolemmal membrane bilayer are altered. A change in the relative fatty acid content of the outer half of the sarcolemmal membrane may alter the lipid environment leading to a decrease in β-receptor-mediated stimulation of adenylate cyclase. In addition the decreased binding capacity of sarcolemmal membrane fragments that we observed may also be due to perturbations of the lipid environment of the membrane. Limbird and Lefkowitz (1976) reported that cleavage of fatty acids from membrane phospholipids with phospholipase A decreased the number but not the affinity of β-receptors from frog erythrocytes. They observed a parallel decrease in catecholamine-stimulated adenylate cyclase. It is conceivable that the cellular–molecular basis for some of the biological actions of PFDA is due to altered fatty acid processing in the body leading to changes in the composition and function of important membrane systems. Olson and Andersen (1983) proposed that due to its fatty acid structure PFDA might interfere with normal fatty acid metabolism and that biological membranes with their high fatty acid content might be potential targets for its toxic effects. Recent investigations provide some experimental support for this proposal. Wigler and Shah (1986) reported that PFDA inactivated a membrane channel for 2-aminopurine in L5178Y mouse lymphoma cells. Levitt and Liss (1986) reported that lethal concentrations of PFDA altered surface membrane expression and immunoglobulin secretion in human and murine β-cell lymphomas. George and Andersen (1986) observed that PFDA partially coeluted with liver phospholipids on thin-layer chromatography and suggested that because of its fatty acid structure PFDA might be incorporated into this lipid fraction thereby altering membrane function. Our results which focus on PFDA-induced changes in cardiac membrane function tend to support the hypothesis of cellular membranes as targets for the toxic effects of PFDA.

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The Effect of Thyroxine Supplementation on the Response to Perfluoro-n-Decanoic Acid (PFDA) in Rats

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Short Title

PFDA, and Thyroxine Supplementation

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Abstract

The effects of thyroxine (T_4) supplementation on PFDA-induced decreases in food consumption, body weight, and body temperature were examined. A dose-response study was carried out with 50, 100, 200 or 250 µg/kg ip doses of T_4 for seven days prior to PFDA administration, and daily dosing with T_4 was continued for an additional 30 days. From this study a T_4 dose of 200 µg/kg was chosen and subsequent experiments were conducted with this dose. Supplementation with 200 µg/kg T_4 daily alleviated the hypophagia but not the severe weight loss and hypothermia produced by PFDA treatment. Our results suggest that some component of the thyroid axis plays a role in feeding behavior. In addition, the PFDA-induced wasting syndrome and hypothermia appear to be unrelated to changes in serum thyroid hormones. The unexpected observation that severe weight loss occurred in the presence of essentially normal food intake suggests that PFDA alters basic cellular metabolic processes.

INTRODUCTION

Perfluoro-n-decanoic acid (PFDA) is a member of a family of perfluorinated carboxylic acids that have found widespread commercial applications as lubricants, surfactants, wetting agents, corrosion inhibitors, and gloss enhancers of commercial aqueous emulsion type floor waxes (Bryce, 1964). Perfluorinated fatty acid surfactants have also been used to impart oil and water repellancy to cloth, leather and paper. They are found in aqueous film-forming foam fire extinguishants (Shinoda and Nomura, 1980).

Toxicity from PFDA exposure has been studied in laboratory animals. In rats a single dose of PFDA has been reported to produce hypophagia and severe weight loss (Olson and Andersen, 1983, Langley and Pilcher, 1985), bradycardia, hypothermia and decreased serum thyroid hormone levels (Langley and Pilcher, 1985). Serum thyroxine levels were significantly lower than control levels as early as 12 hours after a single 75 mg/kg dose of PFDA, and were maximally reduced within 48 hours (Langley and Pilcher, 1985). The hypothermic and myocardial effects of PFDA appeared several days after treatment and required approximately a week to develop fully.

The data indicate that one of the earliest observed responses to a single injection of PFDA is an effect on the levels of thyroid hormones in the circulation. Some of the subsequent effects may in part be secondary to the change in thyroid hormone levels. Accordingly, we investigated the effect of T_4 supplementation on the PFDA-induced changes in body weight, food consumption and body temperature.

METHODS

Groups of male Wistar rats (175-225g) were obtained from Harlan Industries and maintained in a constant-temperature environment (23.3° ± 1°C) for 5 days prior to use. All rats were fed Purina Certified Laboratory Chow # 5008 in pellet form. Unless otherwise indicated all PFDA-treated animals received a single 75 mg/kg ip injection of PFDA in propylene glycol-water (1:1). Animals were sacrificed by decapitation, blood was collected in centrifuge tubes on ice, allowed to coagulate, and centrifuged at 3000 x g for 15 min at 4°C. Serum was stored at -80°C for thyroxine (T_4) and triiodothyronine (T_3) determinations.

Thyroxine Supplementation.

Experiment I.

In order to determine the effect of thyroxine supplementation and to attempt to establish the most effective dose of thyroxine, four groups of rats were pretreated at 8:00 a.m. daily for 7 days prior to PFDA administration with ip injections of 50, 100, 200 or 250 µg/kg of T_4 in saline-1 mM NaOH.Daily T_4 supplementation was continued through the day prior to sacrifice. Body weight changes and food consumption were recorded daily for 30 days after a 75 mg/kg ip injection of PFDA administered at 5:00 p.m. on day 0. Ten rats were used in each treatment group, a group of eight rats received 75 mg/kg PFDA alone, and all animals were sacrificed on day 30. The criteria used to determine the effectiveness of thyroxine supplementation were food consumption, body weight changes and apparent protection from the lethal effects of PFDA. Four of eight rats died following treatment with PFDA alone (one each on days 11, 13, 16 and 17). With 50 µg/kg T_4 6 of 10 rats died following PFDA treatment, (one each on days 12, 13, 16 and 19 and two on day 14). Three of 10 rats died with 100

μg/kg T_4 (two on day 10 and one on day 12), 3 of 10 rats died with 250 μg/kg T_4 (one each on days 8, 10 and 19), 2 of 10 rats died with 200 μg/kg (both on day 12). The number of animals used was insufficient to clearly establish a protective effect for any dose of thyroxine against the lethal effects of PFDA. Although rats supplemented with 250 μg/kg T_4 regained body weight faster than the other groups 200 μg/kg T_4 was chosen as the "best" dose because it was more effective at maintaining food consumption and fewer animals died following PFDA treatment.

Experiment II

A subsequent study was conducted to confirm the observed effects of 200 μg/kg - T_4 plus 75 mg/kg PFDA and to compare these effects to those of 75 mg/kg PFDA alone and 200 μg/kg T_4 alone. Twelve rats received injections of 200 μg/kg of T_4 at 8:00 a.m. each day for 7 days. On the eighth day (designated day 0), 8 T_4-pretreated rats (PFDA-T_4 group) were dosed with 75 mg/kg PFDA (one animal died 12 days after PFDA), 4 T_4-pretreated rats were dosed with propylene-glycol:water (T_4 group) and T_4 supplementation was continued for the duration of the study. Eight rats received a single ip injection of 75 mg/kg PFDA (one animal died 12 days and one 13 days after PFDA). Body weight changes and food consumption were recorded daily and rectal body temperatures were recorded on alternate days for 14 days after PFDA. Data were collected on four control rats two days prior to PFDA and on day zero and day 14. Feces were collected for all animals over each 24 hour period and total dry weights determined.

All animals were sacrificed on a randomized schedule 14 days after PFDA treatment, and blood was collected for thyroid hormone determinations.

Radioimmunoassay. Total T_4 and T_3 were determined by radioimmunoassay using assay tubes coated with antibody to T_4 or T_3 (Diagnostic Products, Los

Angeles, CA). Concentrations of PFDA up to 100 µg/ml did not interfere with the radioimmunoassays for either T_4 or T_3.

Statistical analysis. Statistical differences in thyroid hormone levels among groups were determined using analysis of variance (ANOVA). Duncan's multiple range test was used to determine significant differences at the 0.05 level between control rats and PFDA, PFDA-T_4, or T_4-treated rats.

RESULTS

A dose of 200 µg/kg of T_4 appeared to be the most effective at preventing the hypophagia associated with 75 mg/kg PFDA-treatment (Figure 1A). Essentially no protection was seen with 50 µg/kg of T_4 while doses of 100 µg/kg and 250 µg/kg of T_4 were intermediate between 50 µg/kg and 200 µg/kg in their effects on hypophagia.

Thyroxine supplementation had a differential effect on the body weight and food consumption patterns following treatment with 75 mg/kg PFDA. The dose of T_4 that was most effective at preventing PFDA-induced hypophagia was relatively ineffective at preventing the severe weight loss (Figure 1B).

Figures 2A and 2B compare the effects of treatment with 200 µg/kg T_4 plus PFDA with the effect of treatment with 200 µg/kg T_4 alone or PFDA alone on food consumption and body weight changes. All animals gained weight at the same rate for the seven days prior to dosing with PFDA or propylene glycol. PFDA treatment produced an initial rapid, then gradual, decrease in food consumption. These results are similar to previous reports (Olson and Andersen, 1983; Langley and Pilcher, 1985). Food consumption in rats treated with PFDA alone was significantly less than that in PFDA-T_4 treated animals from day 2 through day 12 after PFDA (Figure 2A). Body

weight decreased dramatically reaching a cumulative loss of 80 grams (35%) by day 10. The T_4-treated rats consumed relatively normal amounts of food and gained weight rapidly adding a total of nearly 120 gms over the 21 day course of the study. Food consumption in PFDA-T_4 rats was similar to control rats. In spite of relatively normal food consumption, weight loss in the PFDA-T_4 treated rats was essentially the same as that in rats treated with PFDA (Figure 2B).

Rectal body temperatures in PFDA-treated rats were significantly lower than in PFDA-T_4-treated rats at two and six days after PFDA. Body temperatures were essentially the same in both groups at other time points (Fig. 3). Rectal body temperatures were the same in all three treatment groups but PFDA and PFDA-T_4 animals were significantly less than control by day 14.

Daily injections of 200 µg/kg thyroxine caused significant elevations in serum T_4 and T_3 levels compared to controls (Table 1). While thyroxine supplementation elevated the serum T_4 levels in the PFDA-T_4 group compared to the PFDA group, serum T_4 levels at day 14 in the PFDA-T_4 group were still significantly less than controls.

DISCUSSION

Supplementing rats with thyroxine (200 µg/kg daily) alleviated the hypophagia but was ineffective at preventing the body weight loss and hypothermia associated with PFDA treatment. The results of this study suggest that some component(s) of the hypothalamus-pituitary-thyroid axis directly or indirectly affects feeding behavior since partial replacement of thyroid hormone levels prevented PFDA-impaired feeding. Evidence has been presented for a role for thyrotropic releasing hormone (TRH) in feeding

behavior through modulation of activity in the ventral medial nucleus of the hypothalamus (VMH). Injections of TRH into the VMH of rats suppressed feeding behavior (Vijayan and McCann, 1977) even in refed starved animals (Suzuki et al., 1982). The fall in serum thyroid hormones produced by PFDA may result in a reflex increase in release (Hirooka, et al. 1978) or enhanced activity of TRH (Hinkle et al., 1981) in the hypothalamus leading to suppression of feeding. These results also demonstrate that the PFDA-induced wasting syndrome cannot be attributed entirely to hypophagia nor to the reduction in serum thyroid hormones. Likewise, PFDA-induced hypothermia is apparently not due to reduced serum thyroid hormones since partial replacement of the thyroid hormones failed to prevent this response.

The dicotomy between food consumption and body weight changes in PFDA-T_4 rats is difficult to explain. There were no significant differences in total fecal excretion between PFDA-T_4 and T_4 treated animals. Metabolic substrates from the diet are normally used by the body to produce energy for catabolic and anabolic activity and these cellular processes evolve heat (Lehninger, 1977). In PFDA-T_4-treated rats dramatic weight loss occurred and thermogenesis was reduced in spite of relatively normal amounts of metabolic substrates from the diet. Treatment with 200 µg/kg T_4 resulted in a modest increase in food consumption and normal body weight gain over the duration of the study. These results suggest that PFDA alters some basic metabolic process(es) apparently resulting in a loss of body mass. The cellular mechanism(s) of these effects remain to be determined.

Although the number of animals used in the study was relatively small, it appears unlikely that the delayed lethality is due to a thyroid mechanism since T_4 supplementation failed to prevent this effect of PFDA. Finally, the observation that serum T_4 levels were significantly decreased by PFDA-

treatment in the presence of T_4 supplementation indicates an inability to retain the administered T_4. This may be due to a change in plasma protein binding or possibly increased metabolism and/or excretion. Such effects could explain the significant reductions in circulating thyroid hormone levels which have been observed in PFDA-treated rats (Langley and Pilcher, 1985). Preliminary work in our laboratory suggests that PFDA displaces ^{125}I-T_4 from rat albumin with an affinity similar to that of cold thyroxine (Gutshall et al, 1986).

References

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TABLE 1
Serum levels of total T_4 and total T_3 14 days after a single i.p. injection of PFDA (75mg/kg).

TREATMENT	N	SERUM T_4 (µg/dl)	SERUM T_3 (ng/dl)
		$\bar{X}$ ± SEM	$\bar{X}$ ± SEM
Control	4	6.00 ± 0.6	90.1 ± 10.1
T_4	4	7.97 ± 0.6[a]	159.0 ± 14.2[a]
PFDA	6	1.94 ± 0.4[b,c]	88.2 ± 11.5
PFDA-T_4	7	3.75 ± 0.4[b]	74.8 ± 9.4

[a] Significantly greater than control, $p < .05$.
[b] Significantly less than control, $p < .05$.
[c] Significantly less than PFDA-T_4, $p < .05$.

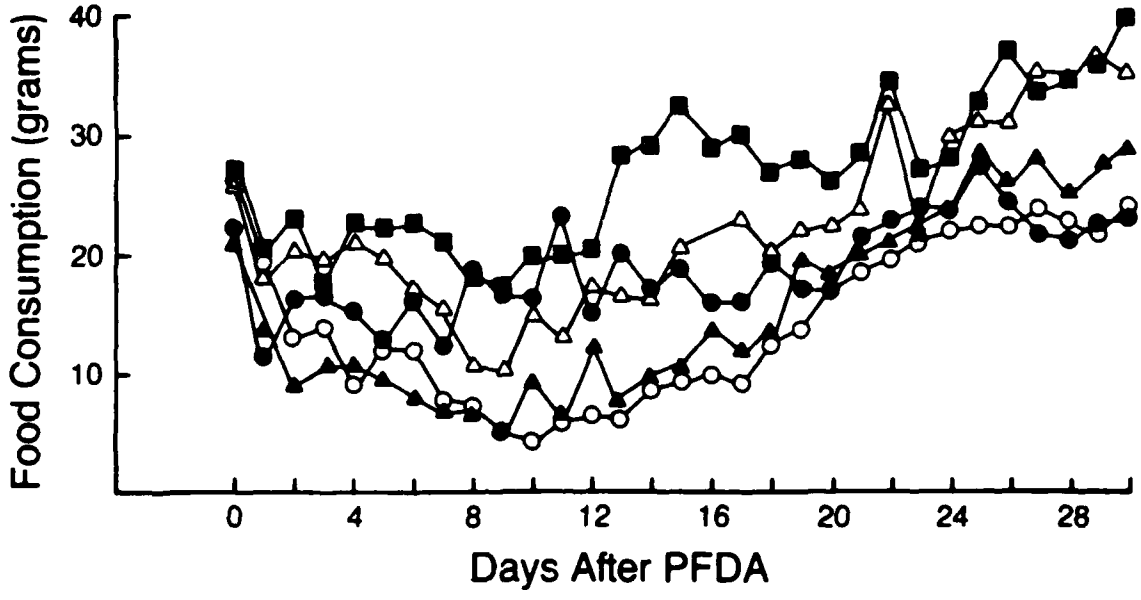
Figure 1A. The effects of thyroxine supplementation on PFDA-induced changes in food consumption. Each point represents the mean daily food consumption (grams). Four groups of rats were pretreated daily for 7 days with ip injections of 50(O, n=10), 100 (Δ, n=10), 200 (■, n=10), or 250 (●, n=10) μg/kg of T_4 before PFDA administration (75 mg/kg) on day 0. T_4 was continued daily until the day prior to sacrifice. PFDA (75 mg/kg, ▲, n=8).

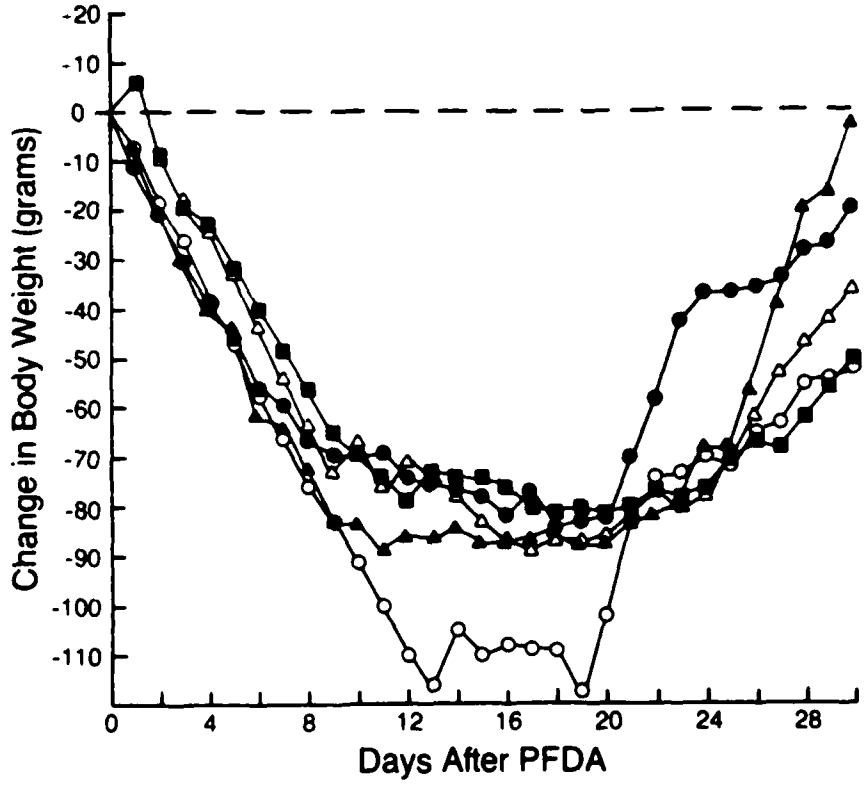
Figure 1B. The effects of thyroxine supplementation on PFDA-induced changes in body weight. Each point represents the mean body weight change (grams) from day 0. Four groups of rats were pretreated daily for 7 days with ip injections of 50 (O, n=10), 100 (Δ, n=10), 200 (■, n=10) or 250 (●, n=10) μg/kg of T_4 before PFDA administration (75 mg/kg) on day 0. T_4 was continued daily until the day prior to sacrifice. PFDA (75 mg/kg, ▲, n=8).

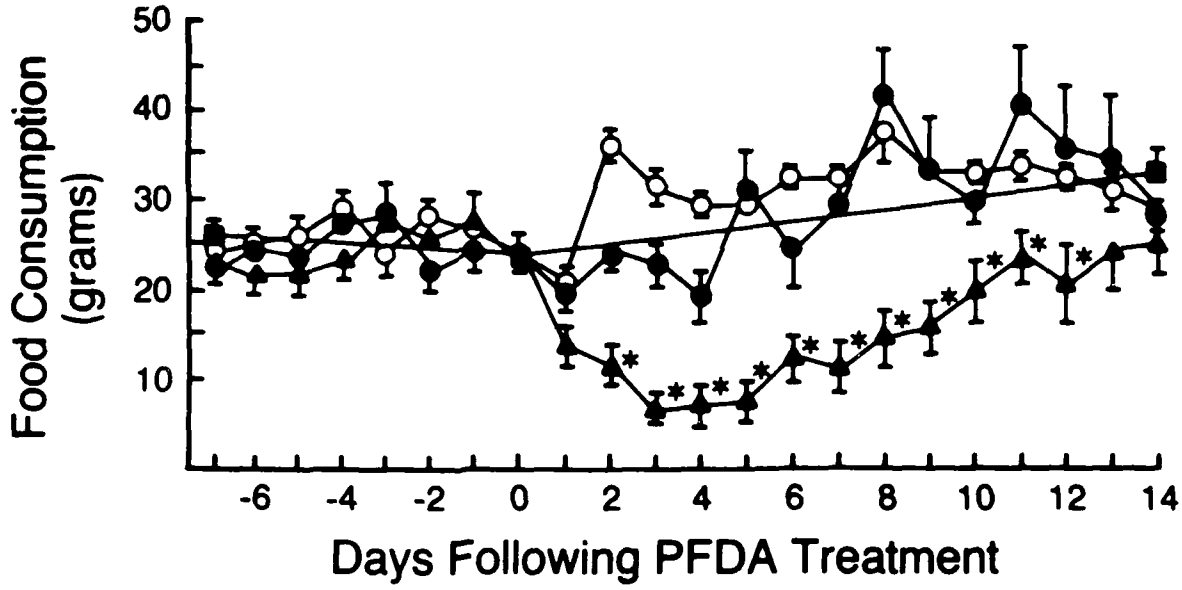
Figure 2A. The effects of PFDA (▲, n=8), T_4 (O, n=4) and PFDA-T_4 (●, n=8) on food consumption. Each point represents the mean daily food consumption (grams). Rats were pretreated daily for 7 days with 200 μg/kg T_4 ip before PFDA administration (75mg/kg) on day 0. T_4 was continued daily through the day prior to sacrifice. Control (■, n=4). *Significantly less than PFDA-T_4, $P < .05$.

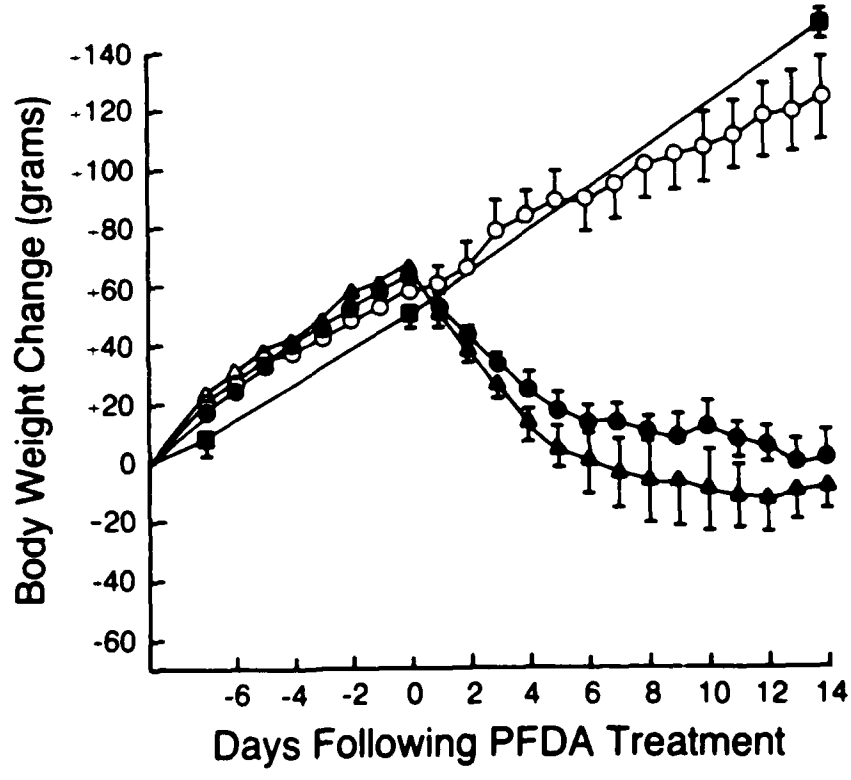
Figure 2B. The effects of PFDA (▲, n=8), T_4 (O, n=4) and PFDA-T_4 (●, n=8) on body weight changes. Each point represents the mean body weight change (grams) from day 0. Rats were pretreated daily for 7 days with 200 μg/kg T_4 ip before PFDA administration (75mg/kg) on day 0. T_4 was continued daily through the day prior to sacrifice. Control (■, n=4).

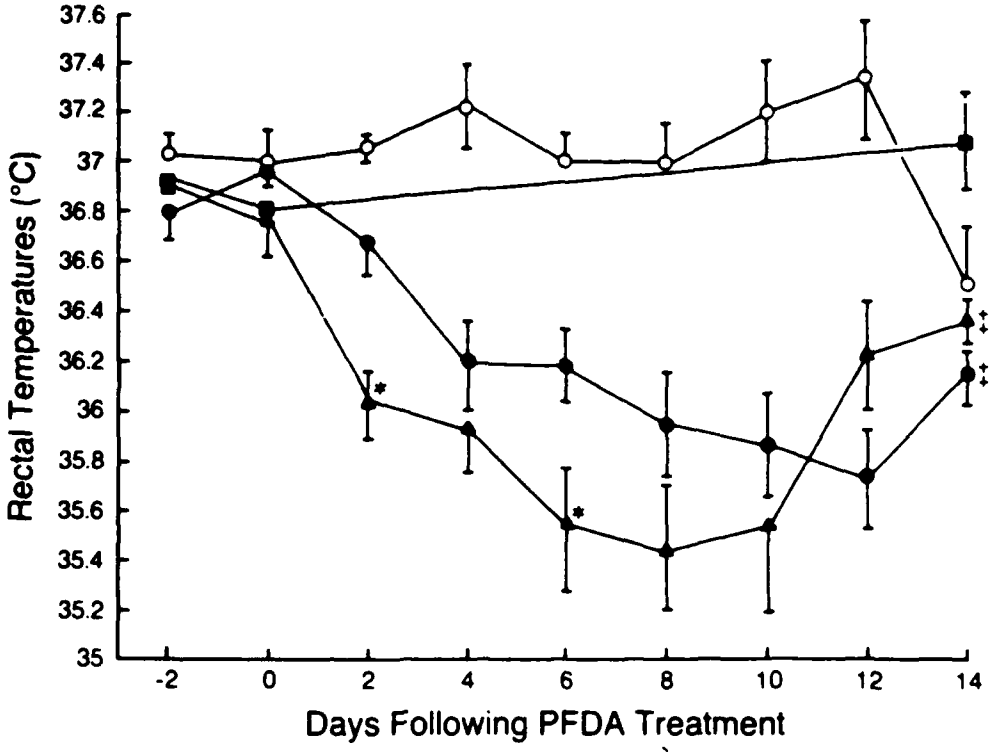
Figure 3. The effects of PFDA (▲, n=8), T_4 (O, n=4) and PFDA-T_4 (●, n=8) on rectal body temperature. Each point represents the mean temperature (°C) $\pm$ SEM. Rats were pretreated daily for 7 days with 200 μg/kg T_4 ip before PFDA administration (75 mg/kg) on day 0. T_4 was continued daily until the rats were sacrificed 14 days later. Control (■, n=4). * Significantly less than PFDA-T_4, $p < .05$. ‡ Significantly less than control, $p < .05$.


Food Consumption (grams)
40
30
20
10
0
4
8
12
16
20
24
28
Days After PFDA


Change in Body Weight (grams)
-20
-10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
0
4
8
12
16
20
24
28
Days After PFDA


Food Consumption (grams)
50
40
30
20
10
-6
-4
-2
0
2
4
6
8
10
12
14
Days Following PFDA Treatment


Body Weight Change (grams)
-140
-120
-100
-80
+60
+40
+20
0
-20
-40
-60
-6
-4
-2
0
2
4
6
8
10
12
14
Days Following PFDA Treatment


Rectal Temperatures (°C)
37.6
37.4
37.2
37
36.8
36.6
36.4
36.2
36
35.8
35.6
35.4
35.2
35
-2
0
2
4
6
8
10
12
14
Days Following PFDA Treatment
*
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‡
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